

NOVEL CYANOENAMINES USEFUL
AS LIGANDS FOR MODULATING GENE EXPRESSION IN
PLANTS OR ANIMALS

AN APPLICATION FOR
UNITED STATES LETTERS PATENT

By

Jonas Grina

Cary, North Carolina

-1- "Express Mail" label number EV023031955US
Date of Deposit FEBRUARY 27, 2002
I hereby certify that this paper or fee is being deposited with
the United States Postal Service "Express Mail Post Office to
Addressee" service under 37 C.F.R. 1.10 on the date indicated
above and is addressed to the Commissioner of Patents and
Trademarks, Washington, D.C. 20231
Bonnie S. Sheridan

Bonnie S. Sheridan

Description

NOVEL CYANOENAMINES USEFUL AS LIGANDS FOR MODULATING GENE EXPRESSION IN PLANTS OR ANIMALS

Cross Reference to Related Applications

The present patent application claims benefit of U. S. Provisional Patent
Application Serial No. 60/272,905, filed March 2, 2001 and is incorporated
herein by reference.

Technical Field

The present invention relates, in general, to novel compounds that are
useful as ligands for modulating gene expression in living organisms (plants
and/or animals). More particularly, the present invention relates to compounds
that are cyanoenamines that are useful as non-steroidal ligands for modulating
exogenous gene expression in eukaryotic organisms (i.e., those where the cell
has a nucleus), more particularly plants, especially chlorophyll-containing
plants.

Table of Abbreviations

n-BuLi

n-butyllithium

	t-butyl	<i>tert</i> -butyl
	C	centigrade
	DMAP	N,N-dimethylaminopyridine
	DNA	deoxyribonucleic acid
5	EcR	ecdysone receptor
	EC80	effective concentration that produces an 80% effect
	GC	gas chromatography
10	GR	glucocorticoid receptor
	g	gram
	Hv	<i>Heliothis virescens</i>
	h	hour
15	L1	first larval instar, namely the stage between the egg and the first molt
	LC	liquid crystal
	LDA	lithium diisopropylamide
20	MS	mass spectroscopy
	mp	melting point
	μ M	micromole
	mL	milliliter
	mm	millimeter
25	min	minute
	M	mole
	NMR	nuclear magnetic resonance

	#	number
	ppm	parts per million
	RNA	ribonucleic acid
	RXR	retinoid X receptor
5	SPODLI	<i>Spodoptera littoralis</i>
	THF	tetrahydrofuran
	USP	ultraspiracle

Background of the Invention

10 Precise temporal control of gene expression is a valuable tool in the field of genetic engineering. The ability to activate (i.e., to induce) or to suppress a gene is of vast importance in manipulating, controlling, and/or studying development and other physiological processes. Inducability is often valuable for foreign protein production, such as production of therapeutic proteins,
15 industrial enzymes, and polymers, in both plants and animals.

 Specifically in the case of plants, often desirable is the control of the timing and level of expression of a phenotypic trait in a plant, plant cell or plant tissue. Ideally, regulation of expression of such a trait can be achieved whenever desired by triggering gene expression with a chemical that is easily
20 applied to field crops, ornamental shrubs and other plants of economic importance. This triggering mechanism for gene expression control is referred to as a gene switch. In order to avoid unexpected activation of the gene switch, the chemical should be one that is normally absent from the plant.

 One such gene switch mechanism is the ecdysone receptor (EcR). EcR

is a member of the nuclear hormone family of receptors. Members of this receptor family are multi-domain proteins, capable of regulating gene expression in response to a chemical ligand. The DNA binding domain (also known as the C domain) binds to a specific target DNA sequence. This
5 specificity determines which target genes are activated by the receptor. The ligand binding domain (E domain) plays a critical role in the determination of ligand specificity as well as the ligand regulated activation property of the receptor. The hinge domain (domain D) resides between the DNA binding and ligand binding domains. The hinge domain modulates the receptor's response
10 to ligand induction.

Ligands that are complementary to the ligand binding domain of the ecdysone receptor are known. Steroidal agonists such as 20-hydroxy ecdysone, muristerone, and ponasterone are capable of activating an ecdysone receptor gene switch. Non-steroidal agonists have advantages over
15 steroidal agonists due to such factors as greater stability, cheaper cost, and environmental acceptance. One known non-steroidal agonist is the insecticide Tebufenozide (also known as the insecticide sold under the trademark MIMIC®).

Of interest is European Published Patent Application No. 0 965 644 A2
20 to Carlson et al., assignors to Rohm and Haas Company, which relates to a method of modulating exogenous gene expression in which an ecdysone receptor complex is contacted with a DNA construct having an exogenous gene under the control of a response element, and where the binding of the

ecdysone receptor to the response element results in activation or suppression of the gene. The ligand is chosen from certain dibenzoyl-*tert*-butyl-hydrazine compounds.

5 As referred to herein, an "ecdysone receptor gene switch" means a gene switch comprising an ecdysone receptor. The ecdysone receptor gene switch may be a heterodimer of EcR and USP, or EcR and RXR. The heterodimerization partner may be native to the organism or cell type in which the gene switch is present, or the heterodimerization partner may be provided
10 exogenously. The ecdysone receptor gene switch may be comprised only of EcR in the absence of a heterodimerization partner. EcR may be in its native form, as isolated from insects, comprising a DNA binding domain, hinge and ligand binding domain from an insect EcR. EcR may be a chimeric protein comprising a DNA binding domain from another EcR or another transcription
15 factor such as Ga14. EcR may comprise its native activation domain or an activation domain of another protein. Furthermore, EcR may comprise a ligand binding domain of an insect ecdysone receptor or a ligand binding domain from a member of the nuclear hormone family of receptors.

 Also of interest is International Publication No. WO 00/15791 to
20 Albertsen et al., assignors to Pioneer Hi-Bred International, Inc. This Publication relates to novel ecdysone receptors from the insect species *Ostrinia* and the genus *Pyralidae* and their use for gene regulation in plants.

 Additionally of interest is International Publication No. WO 99/02683 to Gage et al., assignors to The Salk Institute for Biological Studies. This

Publication relates to nuclear receptor proteins from the silk moth *Bombyx mori*, useful for the regulation of gene expression.

Also of interest is International Publication No. WO 96/37609 to Jepson et al., assignors to Zeneca, relating to the use of a chimeric ecdysone receptor gene switch in plants.

Of general background interest is each of the following describing examples of ecdysone receptor gene switches: No et al., *Proc. Nat'l. Acad. Sci.*, 93: 3346-3351 (1996), describing EcR in mammalian cells; Godowski et al., International Publication No. WO 93/03162, describing EcR and chimeric EcR proteins and related gene switches; Evans et al., International Publication Nos. WO 99/58155 and WO 97/38117, describing EcR and chimeric EcR proteins and related gene switches; Martinez et al., *Insect. Biochem. Mol. Biol.*, 29 (10):915-930 (October, 1999), describing a chimeric EcR gene switch in plants; Martinez et al., *Plant J.*, 19(1):97-106 (July, 1999), describing a chimeric EcR gene switch in plants; Martinez et al., *Mol. Gen. Genet.*, 261(3):546-552 (April, 1999), describing a chimeric EcR gene switch in plants; Suhr et al., *Proc. Nat'l. Acad. Sci. U.S.A.*, 95(14):7999-8004 (July 7, 1998), describing a chimeric EcR switch in mammalian cells; and Hoppe et al., *Mol. Ther.*, 1(2):159-164 (February, 2000), describing an adenovirus mediated EcR gene switch.

Especially of interest is U.S. Patent No. 5,880,333 to Goff et al., assignors to Novartis Finance Corporation. This patent discloses a method of controlling gene expression in plants. Specifically, the method involves obtaining a transgenic plant that has at least 2 receptor expression cassettes

and at least 1 target expression cassette. A first of the 2 receptor expression cassettes has a nucleotide sequence for a 5' regulatory region operably linked to a nucleotide sequence that encodes a first receptor polypeptide and a 3' termination region. A second of the 2 receptor expression cassettes has a nucleotide sequence for a 5' regulator region operably linked to a nucleotide sequence that encodes a second receptor polypeptide and a 3' termination region. The target expression cassette has a nucleotide sequence operably linked to a nucleotide sequence that encodes a target polypeptide and a 3' termination region, wherein the 5' regulatory region of the target expression cassette is activated by the first and second receptor polypeptides in the presence of a certain chemical ligand that is complimentary to the ligand binding domain of the receptor polypeptides, as a result of which expression of the target polypeptide is accomplished. In a preferred embodiment, the method involves expressing in a plant an insect EcR and a second receptor as a heterodimerization partner and activating the expression of a target polypeptide by contacting the plant cells with a ligand that is complimentary to the ligand binding domain of one of the receptors. The method of U.S. Patent No. 5,880,333 to Goff et al. is useful for controlling various traits of agronomic importance, such as plant fertility.

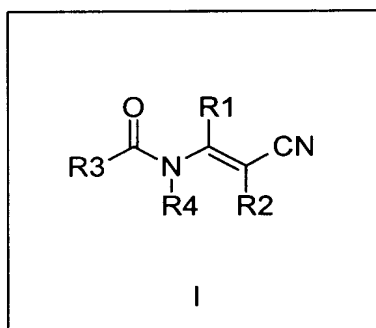
Lastly, of interest is U.S. Provisional Application No. 60/242,969, filed October 24, 2000, describing novel ecdysone receptor gene switches and methods of use, the disclosure of which is incorporated in its entirety.

All of the patents and published patent applications mentioned here are incorporated by reference.

Despite the plethora of available ecdysone receptor gene switch systems, there still remain a continuing need to develop non-steroidal ligands with increased activity as compared to known ligands and a need to develop ligands that demonstrate improved consistent activity in intact plants and animals.

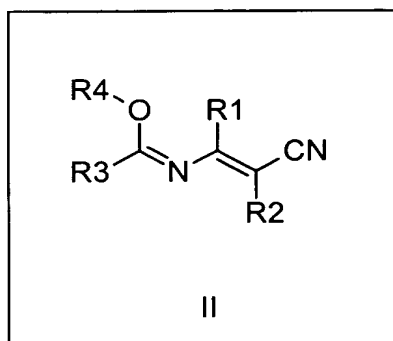
Summary and Objects of the Invention

Accordingly, the present invention provides a compound comprising Formula I

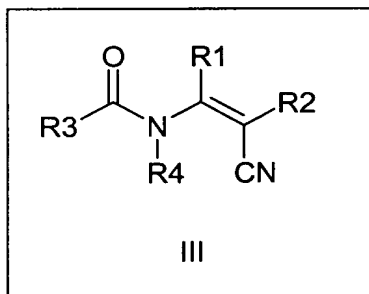


10

and also, Formula I may be in its tautomeric form comprising Formula II



and also, Formula I may be in its isomeric form comprising Formula III



5 wherein:

R1

is a branched chain lower alkyl (C3 to C8), cycloalkyl (C3 to C8), alkyl-substituted alkyl (C4 to C8), bicycloalkyl, 1-adamantyl, polyhaloalkyl, trialkylsilyl, unsubstituted phenyl or
10 optionally substituted phenyl;

R2 and R3

are independently unsubstituted or substituted aromatic
rings, chosen from phenyl, pyridyl, pyrimidinyl, furyl,
15 thiophenyl, pyrazinyl, pyrrolyl, pyrazolyl, 1,2,4-triazolyl,
naphthyl, fluorenonyl, xanthenyl, 4-oxo-1,4-dihydro-
(1,8)naphthyridinyl, thiazolyl, isothiazolyl, 1,3,4-thiadiazolyl,
benzo-1,2,3-thiadiazolyl, oxazolyl, imidazolyl, quinolinyl, or
isoquinolinyl, where a substituent on the rings is one or more

chosen independently from hydrogen, alkyl (C1 to C4),
alkoxy, alkoxyalkyl, hydroxy, amino, alkylamino, dialkylamino,
acylamino, halo, haloalkyl, hydroxyalkyl, dihydroxyalkyl,
alkoxycarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl,
5 unsubstituted or substituted alkylphenyl, unsubstituted or
substituted phenyl, unsubstituted or substituted phenoxy,
nitro, cyano, alkylthio, alkylsulfonyl, aminoalkyl, carboxyalkyl,
or sulfonylalkyl;

and

10 R4

is hydrogen, alkylthio, alkylthioalkyl, alkyloxyalkyl,
acyloxyalkyl, alkyl, acyl, trialkylsilyl, or cyclized together with
R3 and the O in Formula II to form a lactone.

15 The compounds described in the above paragraph are useful for
modulation of an exogenous gene in a living organism. The compounds are
also useful for the control of pests, such as arthropods, parasites, and the like,
by acting as agonists of 20-hydroxyecdysone, the molting hormone.

Therefore, it is an object of the present invention to provide a compound
20 that has the ability to activate or to suppress an exogenous gene.

It is another object of the present invention to provide a compound that
is useful as a pesticide.

Some of the objects of the invention having been stated, other objects will become evident as the description proceeds, when taken in connection with the laboratory examples described below.

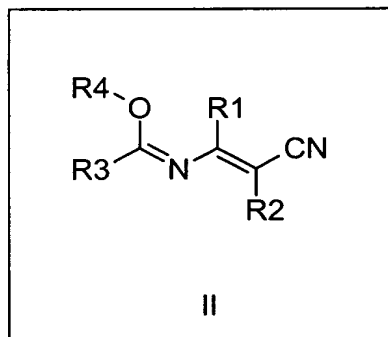
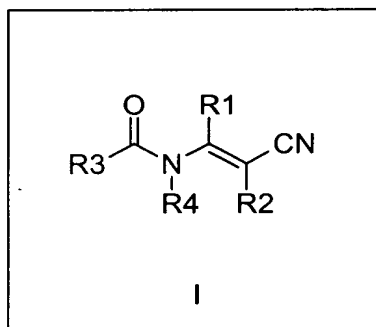
5

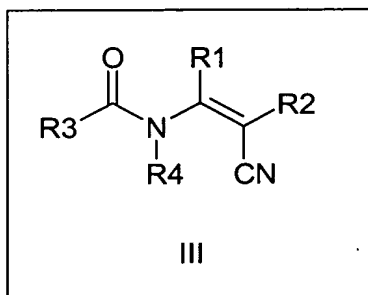
Detailed Description of the Invention

The inventive ligand is an addition to the ligands described in the above-noted U.S. Patent No. 5,880,333 to Goff et. al.

A ligand according to the present invention is described by the below recited general Formula I and its below recited tautomer, Formula II, and its

10 below recited isomer, Formula III:





wherein:

R1

5 is a branched chain lower alkyl (C3 to C8), cycloalkyl (C3 to C8), alkyl-substituted alkyl (C4 to C8), bicycloalkyl, 1-adamantyl, polyhaloalkyl, trialkylsilyl, or optionally substituted phenyl;

R2 and R3

10 are independently optionally substituted aromatic rings, such as phenyl, pyridyl, pyrimidinyl, furyl, thiophenyl, pyrazinyl, pyrrolyl, pyrazolyl, 1,2,4-triazolyl, naphthyl, fluorenonyl, xanthenyl, 4-oxo-1,4-dihydro-(1,8)naphthyridinyl, thiazolyl, isothiazolyl, 1,3,4-thiadiazolyl, benzo-1,2,3-thiadiazolyl, oxazolyl, imidazolyl, quinoliny, or isoquinoliny. Substituents
15 on these rings can be one or more chosen independently from hydrogen, alkyl (C1 to C4), alkoxy, alkoxyalkyl, hydroxy, amino, alkylamino, dialkylamino, acylamino, halo, haloalkyl, hydroxyalkyl, dihydroxyalkyl, alkoxycarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, (optionally substituted) alkylphenyl, (optionally

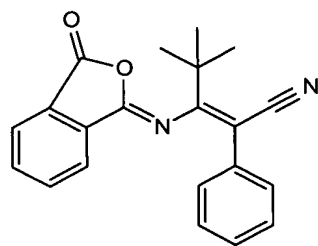
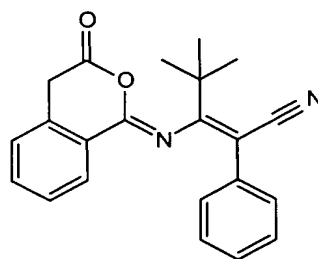
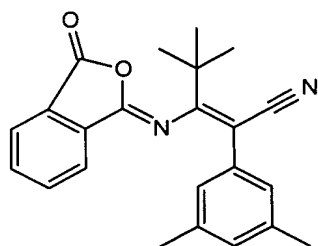
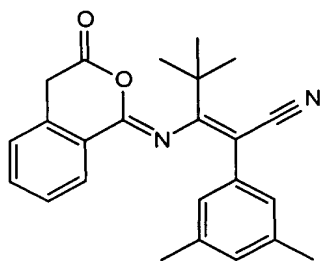
substituted) phenyl, (optionally substituted) phenoxy, nitro, cyano, alkylthio, alkylsulfonyl, aminoalkyl, carboxyalkyl, and sulfonylalkyl;

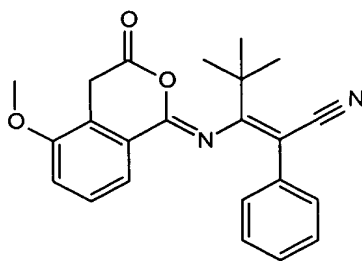
and

R4

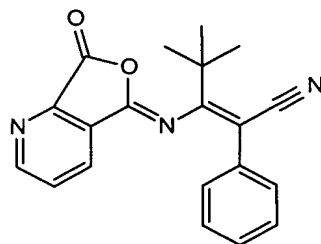
- 5 is hydrogen, or a substituent that may be easily removed *in planta*, serving as an aid in absorption and/or translocation, such as alkylthio, alkylthioalkyl, alkyloxyalkyl, acyloxyalkyl, alkyl, acyl, trialkylsilyl, or cyclized together with R3 and the O in Formula II to form a lactone.

- Halo may be selected from the group consisting of fluoro, chloro, bromo, iodo, and combinations thereof. The substituents on R2 and R3 may also be joined to form cyclic structures on adjacent atoms of the aromatic ring, such as 1,2-methylenedioxy and 1,2-difluoromethylenedioxy. The preferred R1 is *tert*-butyl. The preferred R2 is phenyl, 3,5-dimethylphenyl, 2,4-dimethylphenyl, 3-methylphenyl, 4-methylphenyl, 2-methylphenyl, or 3,4-methylenedioxyphenyl.
- 15 The preferred R3 is phenyl, 3-pyridyl, 3-methoxy-2-methylphenyl, 3-ethoxy-2-methylphenyl, 3-methoxy-2-ethylphenyl, 4-ethylphenyl, 2,6-difluorophenyl, 2,3-dimethylphenyl, 3-chloro-2-methylphenyl, or 3-bromo-2-methylphenyl. When R3, R4, and O are cyclized to form the cyclic ester known as a lactone, the lactone may be:





or



The compounds described as per Formula I and its tautomer, Formula II, and its isomer, Formula III, are useful for modulation of an exogenous gene in a living organism. They have the ability to activate or to suppress an

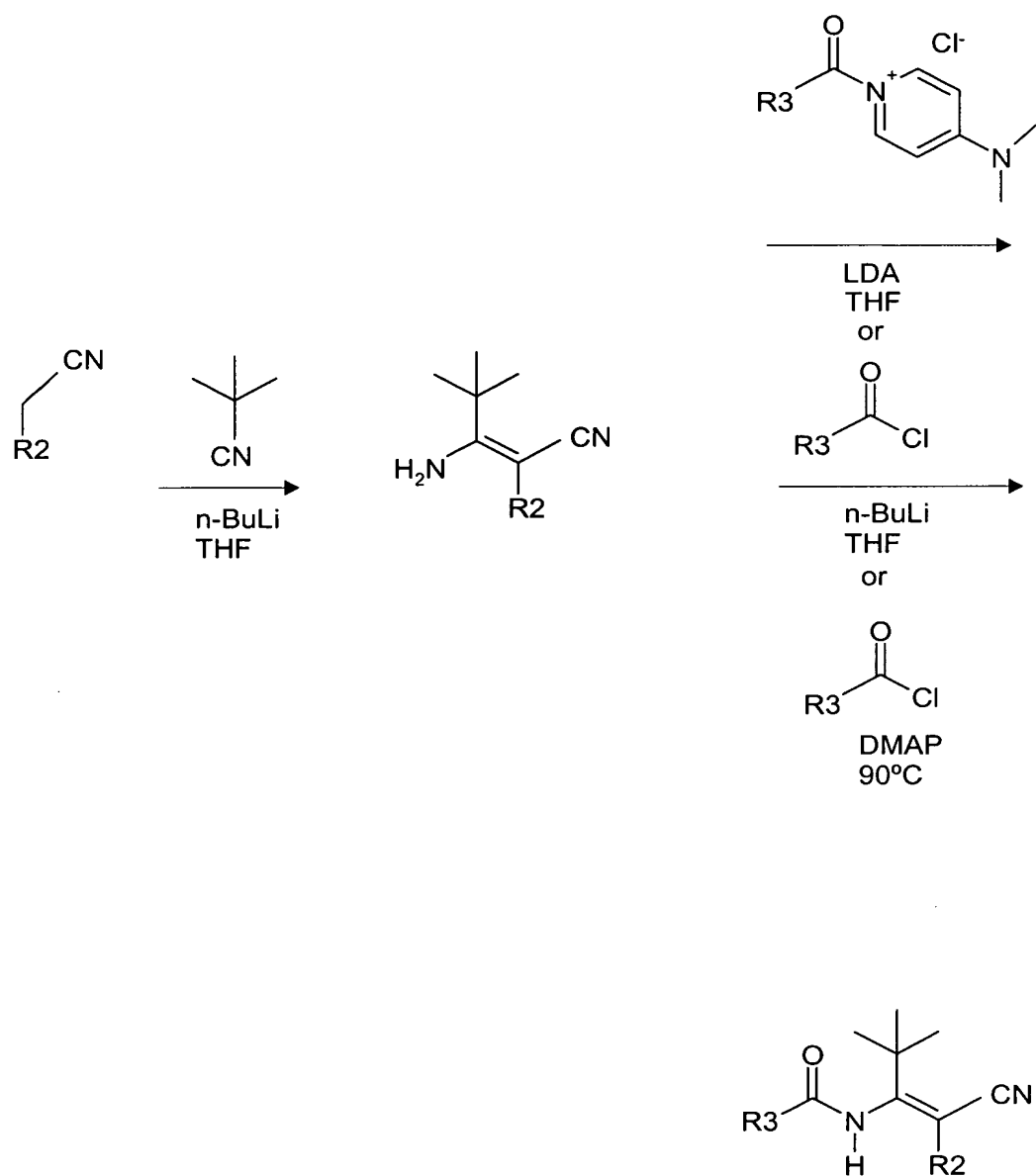
5 exogenous gene.

Additionally, the described compounds can be used as pesticides, i.e., for arthropod control (control of segmented invertebrates such as insects, arachnids, crustaceans, or myriapods) on plants in soil or water, in structures, and on parasites in or on vertebrate animals, acting as agonists of 20-

10 hydroxyecdysone, the molting hormone.

The preparation of these compounds may be accomplished by the

following general scheme:



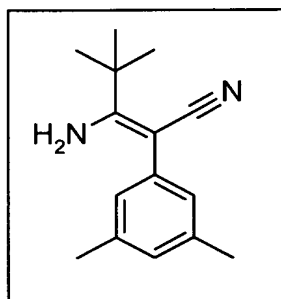
and in this scheme, R1 is *tert*-butyl, but that is not a requirement.

LABORATORY EXAMPLES

Example 1:

Preparation of starting material; (E)-3-amino-2-(3,5-dimethylphenyl)-4,4-

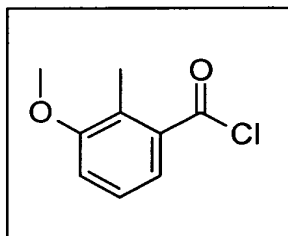
5 dimethylpent-2-enenitrile



To 30 mL of *n*-butyllithium (2.5 M in hexane) at 5°C was added 30 mL of dry tetrahydrofuran. The resulting solution was cooled back to 5°C and a solution of 5 g of 3,5-dimethylphenylacetonitrile in 10 mL of tetrahydrofuran was added over 30 min., keeping the temperature between 5°C and 10°C. The mixture was stirred at 5°C for 1 h., and then a solution of 2.86 g of trimethylacetonitrile in 10 mL of tetrahydrofuran was added. The resulting mixture was stirred overnight at ambient temperature. The mixture was poured into ice water and extracted with 2 portions of ethyl acetate.

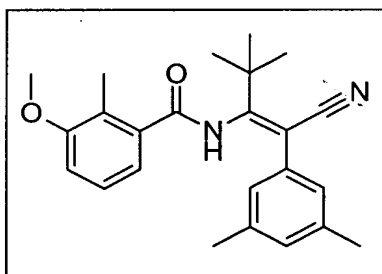
15 The combined ethyl acetate layers were washed with brine, dried over MgSO₄, filtered, and evaporated *in vacuo* to afford the crude product as an oil, which was crystallized from petroleum ether to yield 3.2 g of a solid with a ¹HNMR spectrum consistent with the expected product, namely (E)-3-amino-2-(3,5-dimethylphenyl)-4,4-dimethylpent-2-enenitrile.

Preparation of starting material; 3-methoxy-2-methylbenzoyl chloride



- 5 Thionyl chloride (5 mL) was gradually added to 0.95 g of 3-methoxy-2-methylbenzoic acid at room temperature. The resulting mixture was heated at 65°C for 1 h. The excess thionyl chloride was evaporated *in vacuo*, and a small portion of carbon tetrachloride was added. Then, the mixture was again evaporated *in vacuo* to yield the desired product as an oil. This was used
- 10 directly in the next reaction.

Preparation of N-[(E)-1-*tert*-butyl-2-cyano-2-(3,5-dimethylphenyl)-vinyl]-3-methoxy-2-methylbenzamide



- 15 To 3.8 mL of lithium diisopropylamide solution (1.5 M in cyclohexane) at

-78°C, was added dropwise, a solution of 0.51 g of (E)-3-amino-2-(3,5-dimethylphenyl)-4,4-dimethylpent-2-enenitrile in 30 mL of dry tetrahydrofuran. The mixture was stirred for 30 min. at -78°C, and then 1.05 g of 3-methoxy-2-methylbenzoyl chloride was added in one portion. The resulting mixture was stirred overnight at ambient temperature. The reaction mixture was poured into ice water and extracted with ethyl acetate.

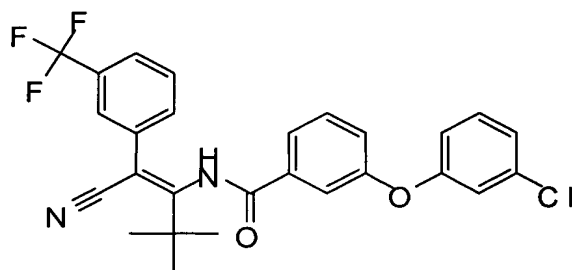
The ethyl acetate layer was washed with brine, dried over MgSO₄, filtered, and evaporated *in vacuo* to yield 1.4 g of crude solid product. The crude product was partially purified using 3 plates, each being a 600 mm x 20 mm silica gel preparative layer chromatography plate, eluted with 20% ethyl acetate in hexane to afford 0.17 g of slightly impure material. This was recrystallized from a 3 mL tetrahydrofuran and 15 mL hexane mixture to yield 0.15 g of white crystalline material (mp was 203 to 204 °C) with GC/MS and ¹HNMR spectra consistent with the desired product, namely N-[(E)-1-*tert*-butyl-2-cyano-2-(3,5-dimethylphenyl)-vinyl]-3-methoxy-2-methylbenzamide.

Example 2:

Using essentially the same procedure as described above, the following selected cyanoenamine compounds have also been prepared, as reported in Table A1 below.

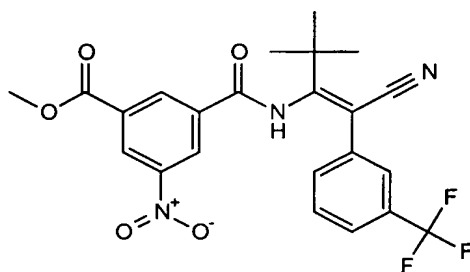
Table A1

Compound	Melting point degrees C	LC/MS molecular ion
----------	----------------------------	------------------------



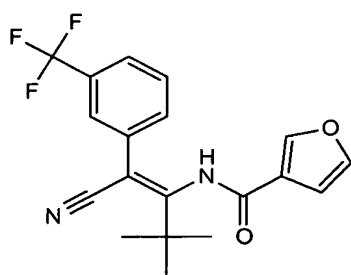
499

Compound 1



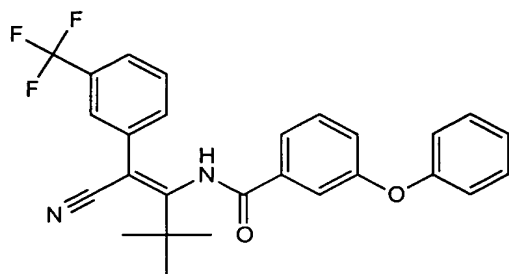
476

Compound 2



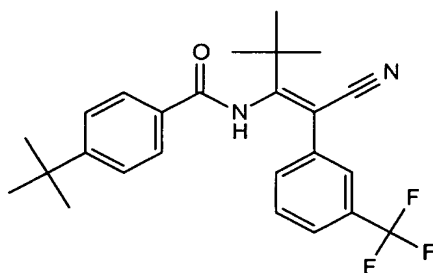
363

Compound 3



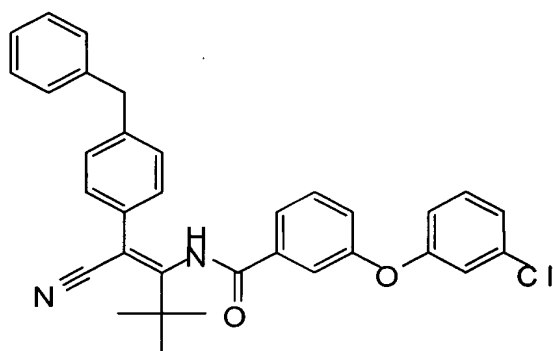
465

Compound 4



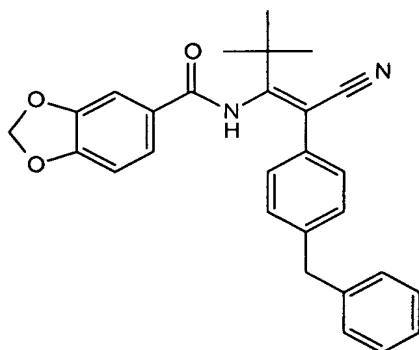
429

Compound 5



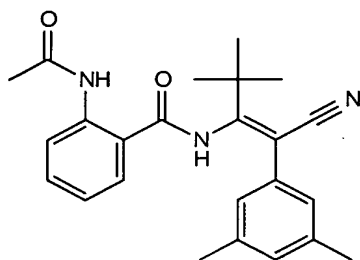
521

Compound 6



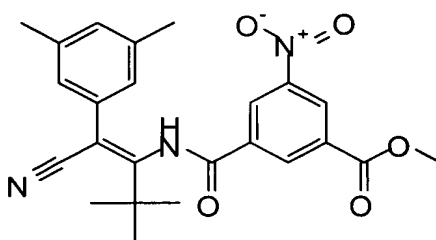
439

Compound 7



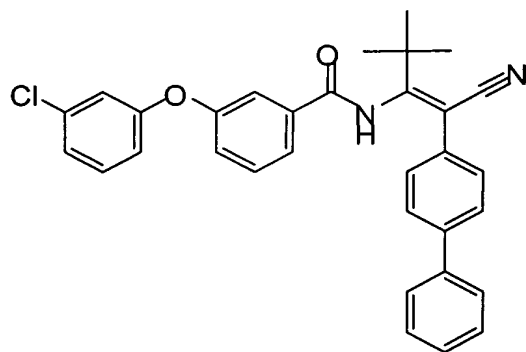
390

Compound 8



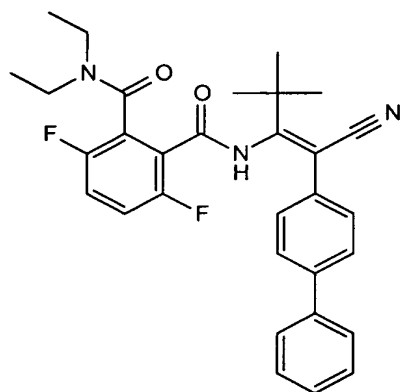
436

Compound 9



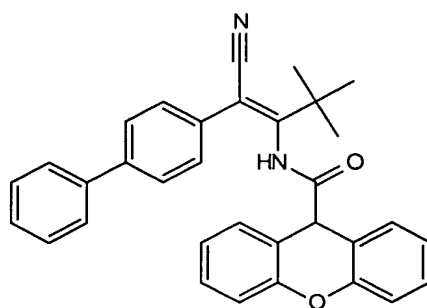
507

Compound 10



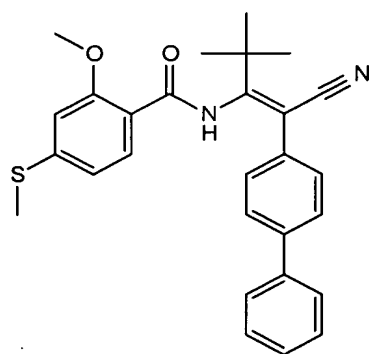
516

Compound 11



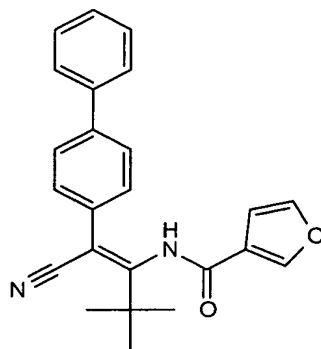
485

Compound 12



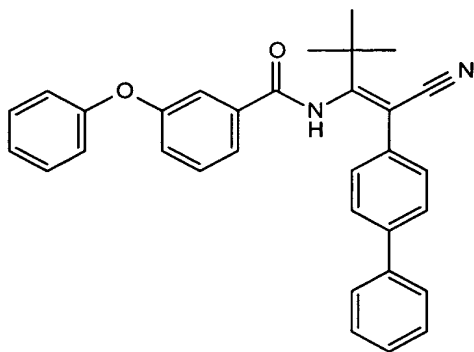
457

Compound 13



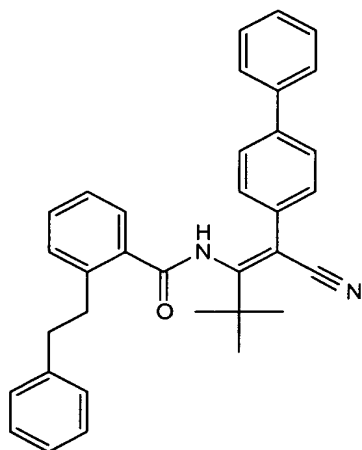
371

Compound 14



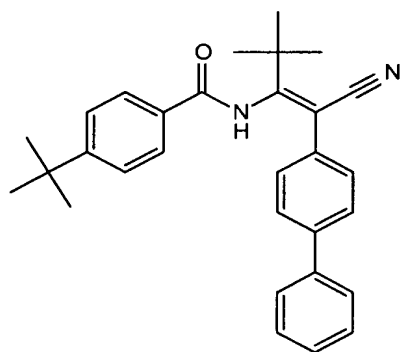
473

Compound 15



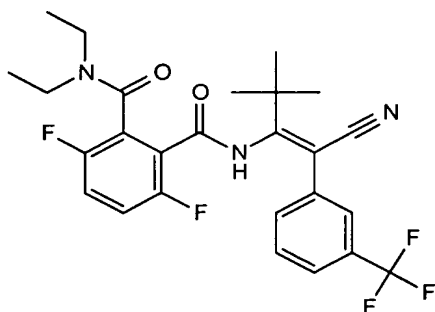
485

Compound 16



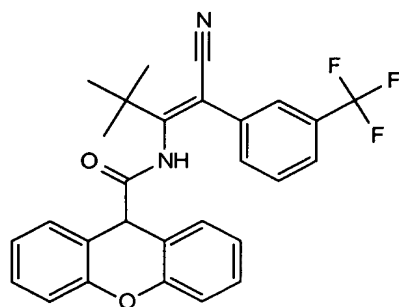
437

Compound 17



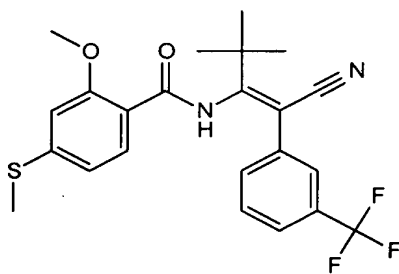
508

Compound 18



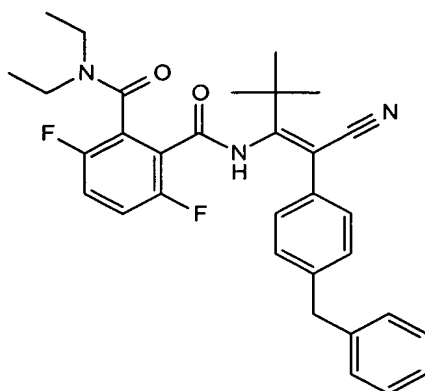
477

Compound 19



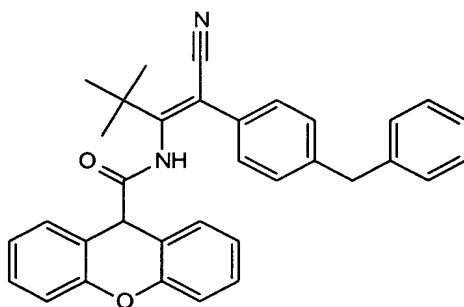
449

Compound 20



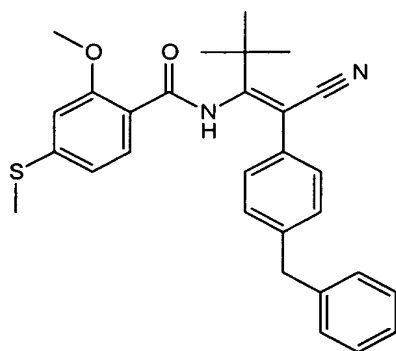
530

Compound 21



499

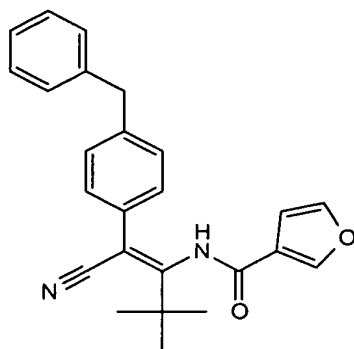
Compound 22



471

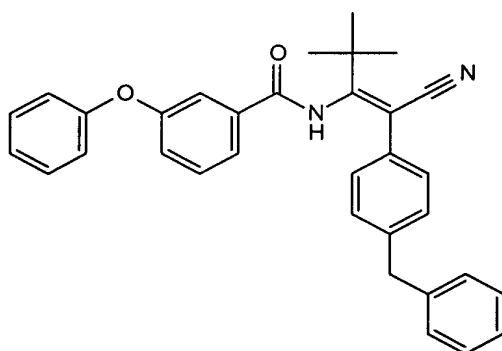
Compound 23

20230929 14:03:03



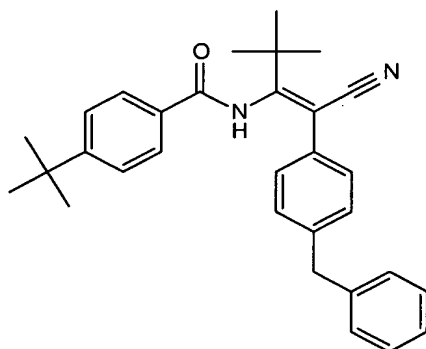
385

Compound 24



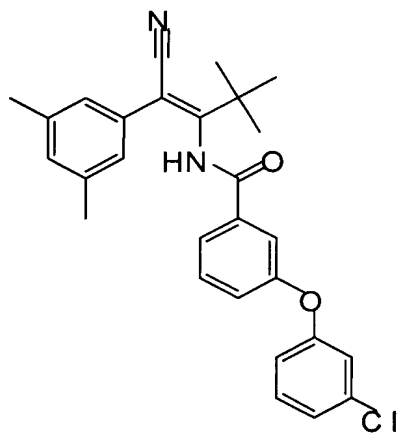
487

Compound 25



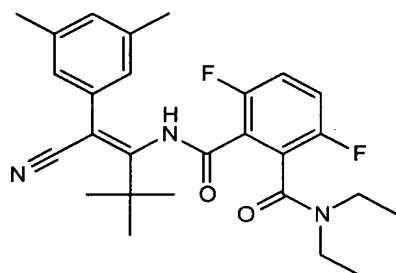
451

Compound 26



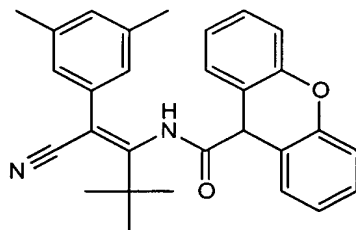
459

Compound 27



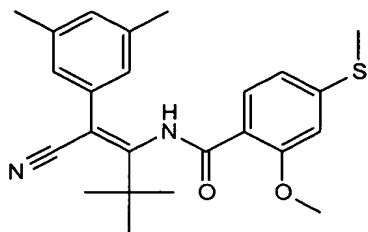
468

Compound 28



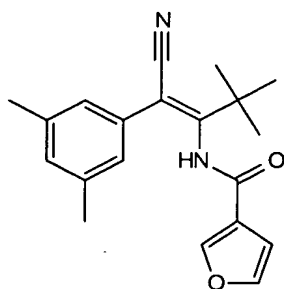
437

Compound 29



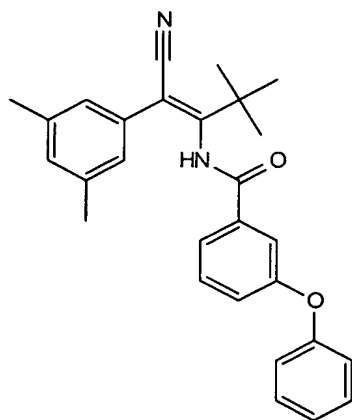
409

Compound 30



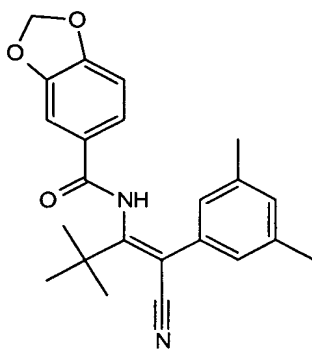
323

Compound 31



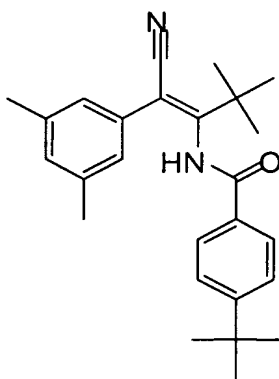
425

Compound 32



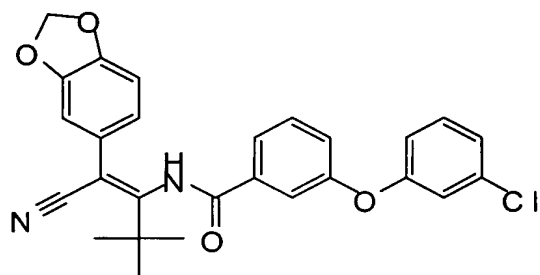
377

Compound 33



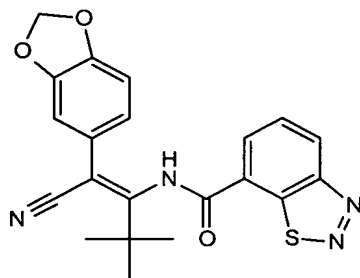
389

Compound 34



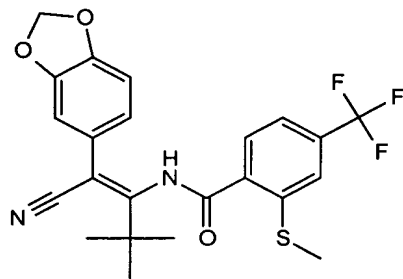
475

Compound 35



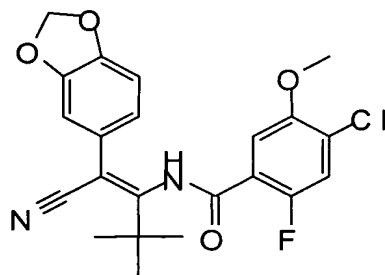
407

Compound 36



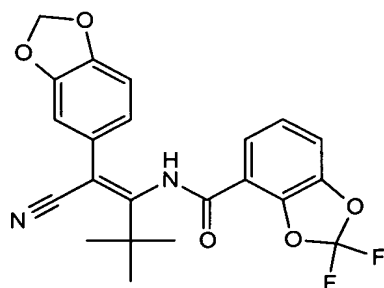
463

Compound 37



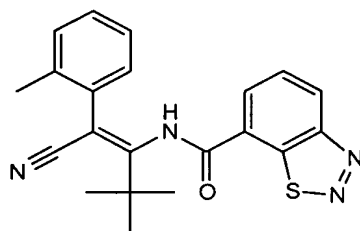
431

Compound 38



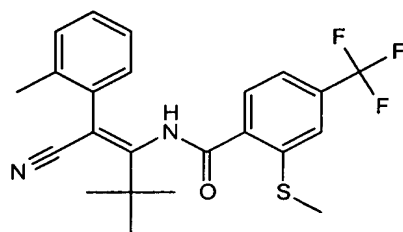
429

Compound 39



377

Compound 40



433

Compound 41



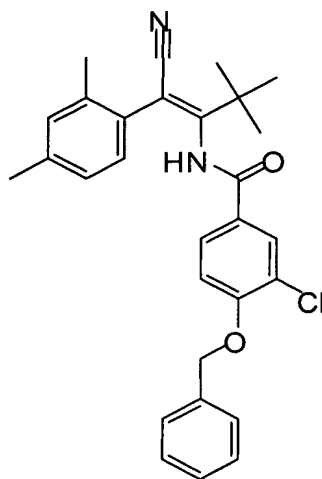
Compound 43



Compound 43

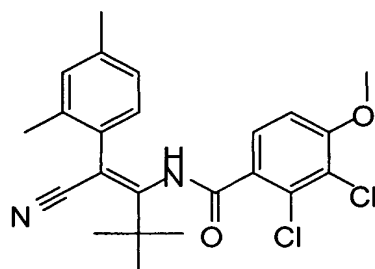


Compound 44



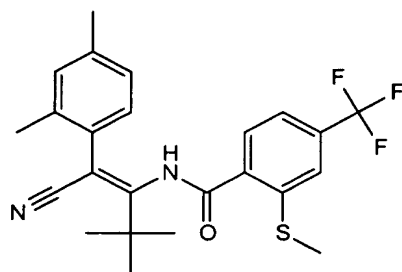
473

Compound 45



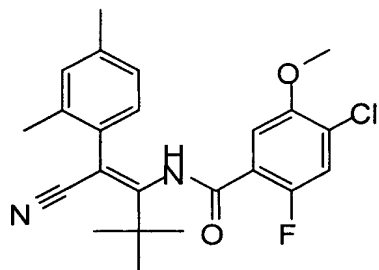
431

Compound 46



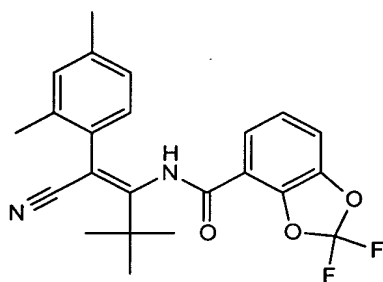
447

Compound 47



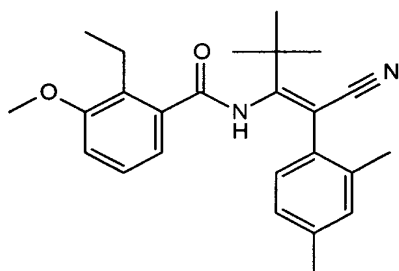
415

Compound 48



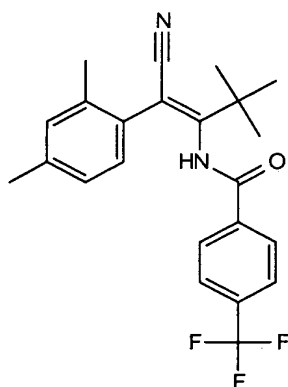
413

Compound 49



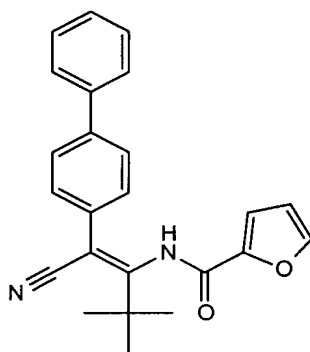
391

Compound 50



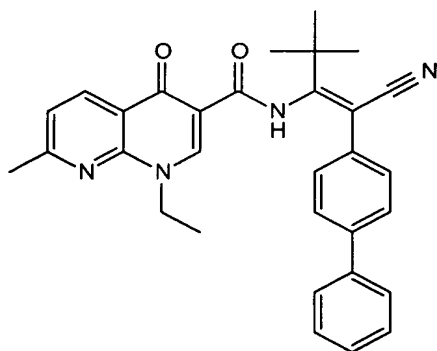
401

Compound 51



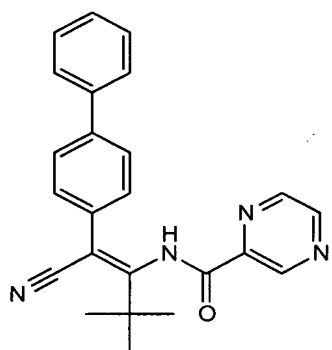
371

Compound 52



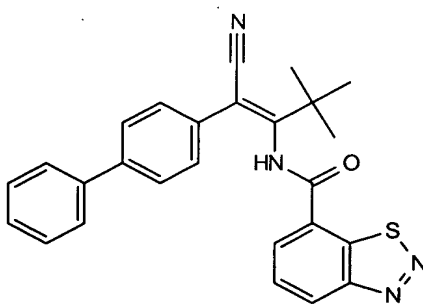
491

Compound 53



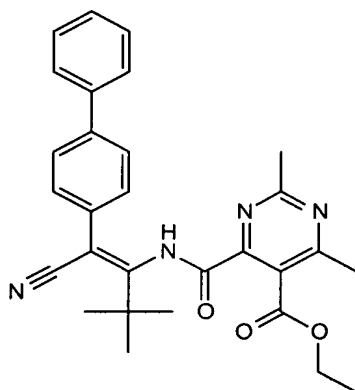
383

Compound 54



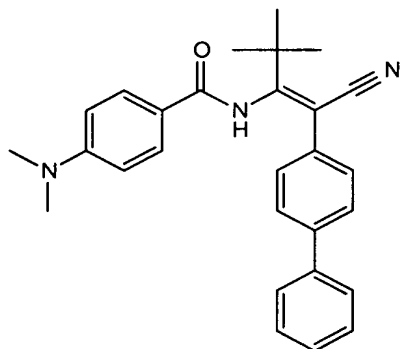
439

Compound 55



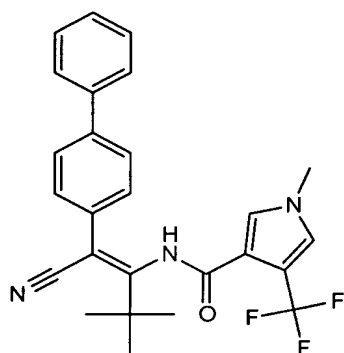
483

Compound 56



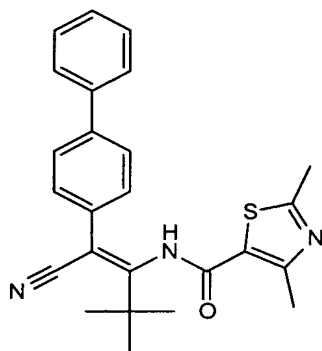
424

Compound 57



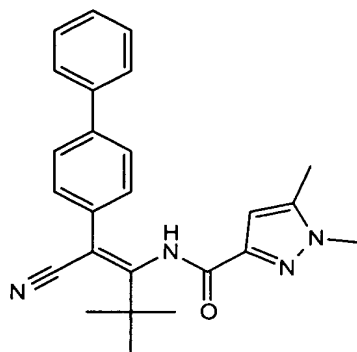
452

Compound 58



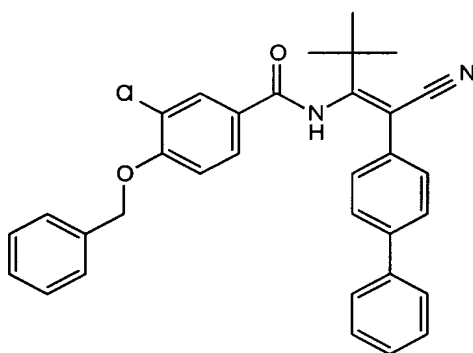
416

Compound 59



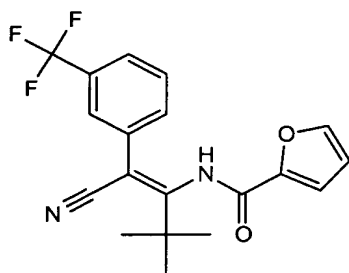
399

Compound 60



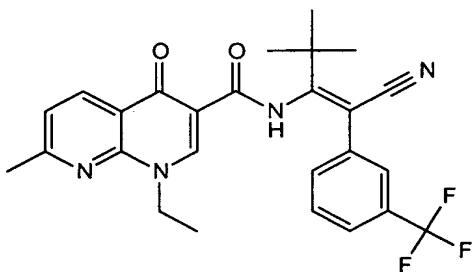
521

Compound 61



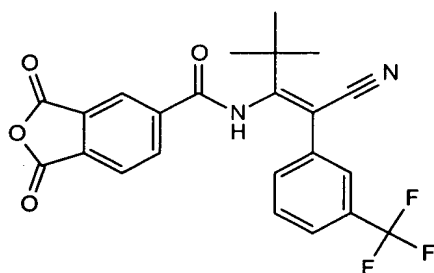
363

Compound 62



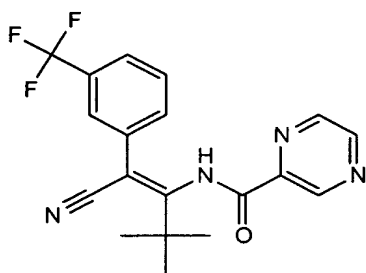
483

Compound 63



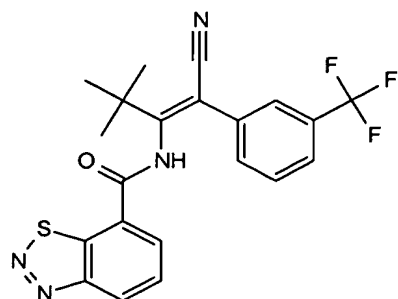
443

Compound 64



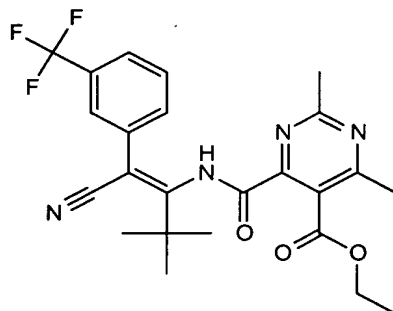
375

Compound 65



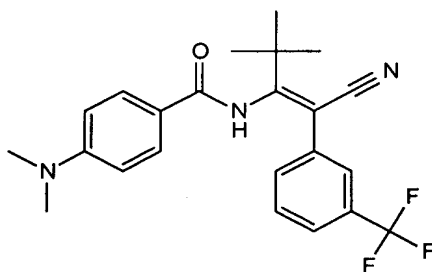
431

Compound 66



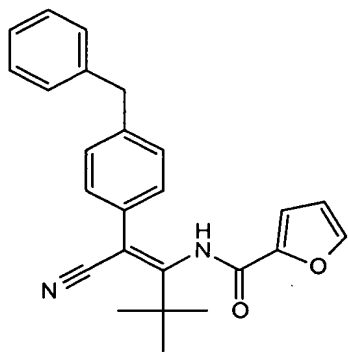
475

Compound 67



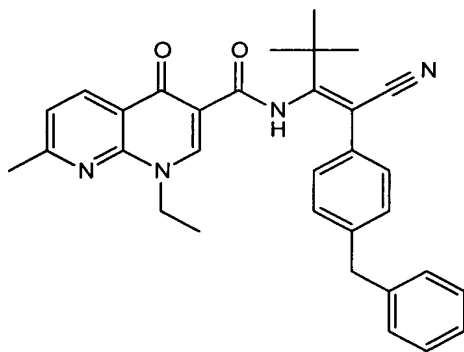
416

Compound 68



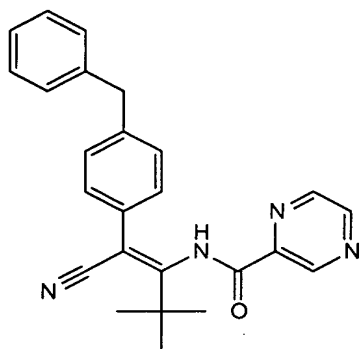
385

Compound 72



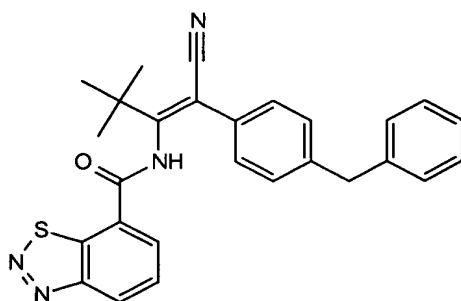
505

Compound 73



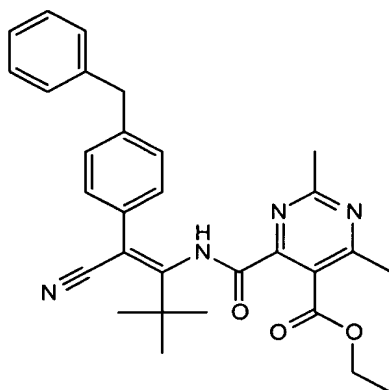
397

Compound 74



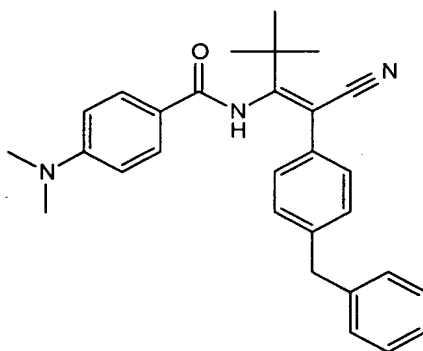
453

Compound 75



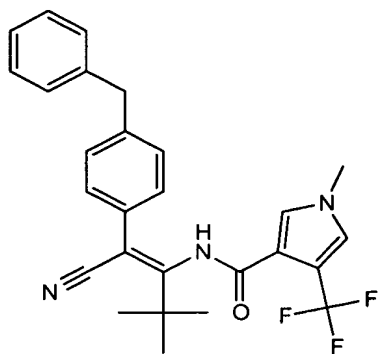
497

Compound 76



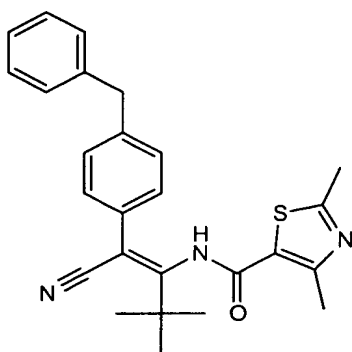
438

Compound 77



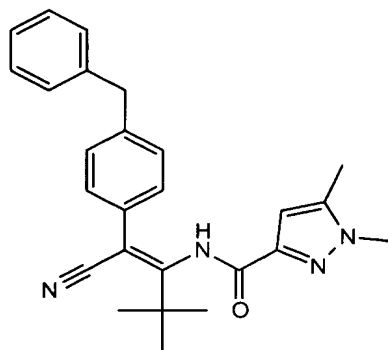
466

Compound 78



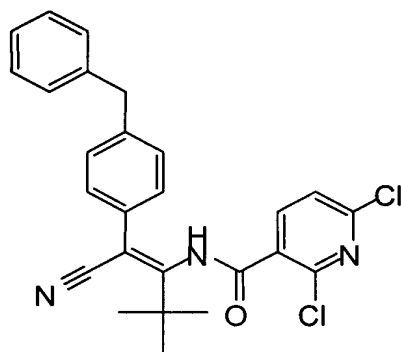
430

Compound 79



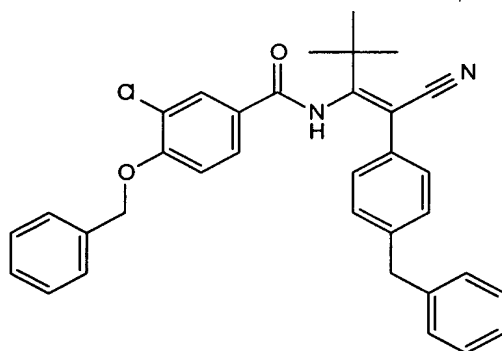
413

Compound 80



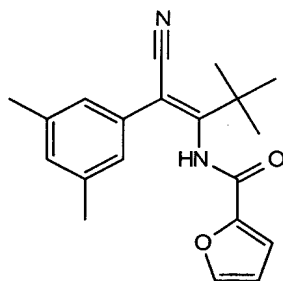
464

Compound 81



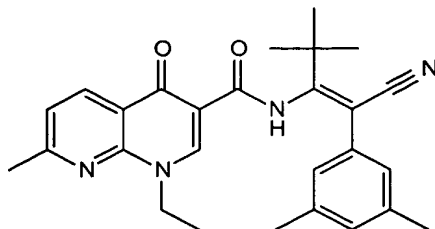
535

Compound 82



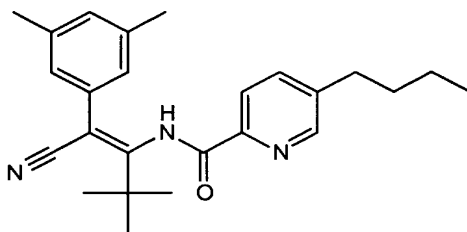
323

Compound 83



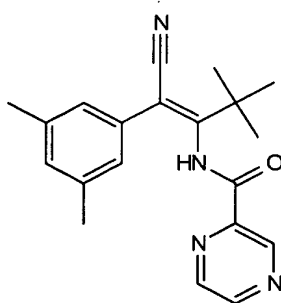
443

Compound 84



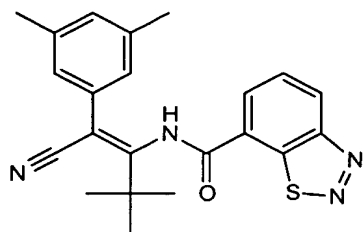
390

Compound 85



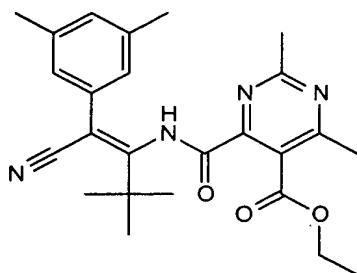
335

Compound 86



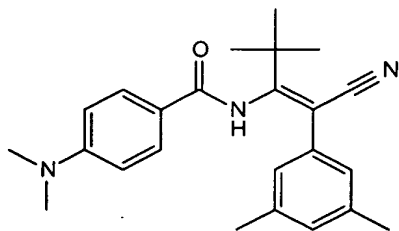
391

Compound 87



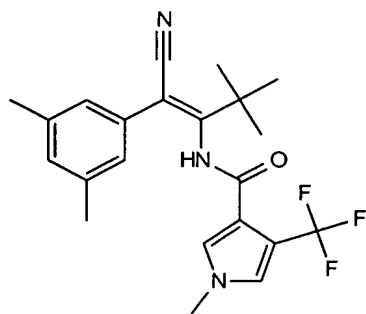
435

Compound 88



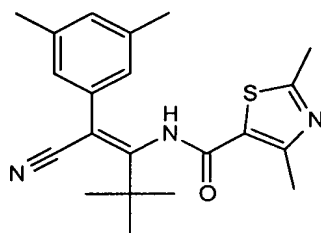
376

Compound 89



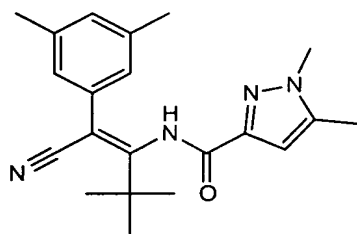
404

Compound 90



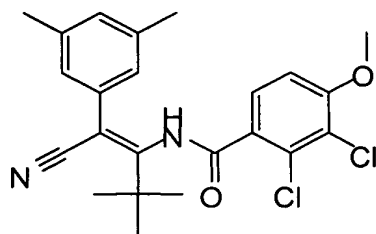
368

Compound 91



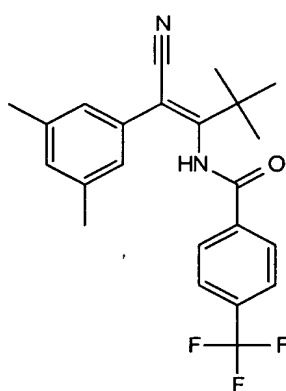
351

Compound 92



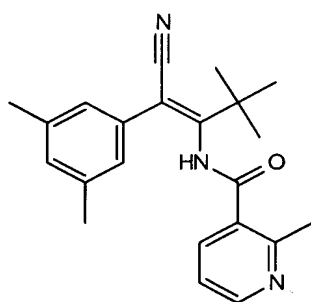
431

Compound 93



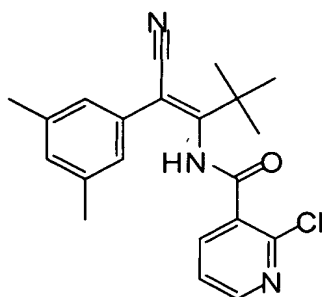
401

Compound 94



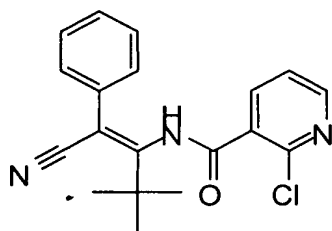
120

Compound 95



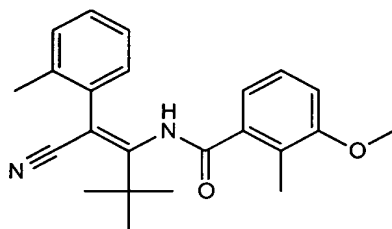
165

Compound 96



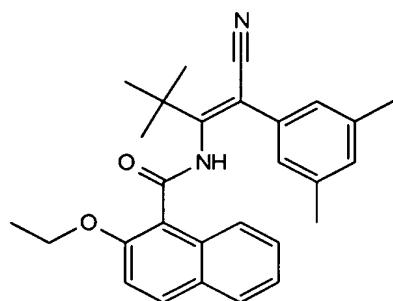
170

Compound 97



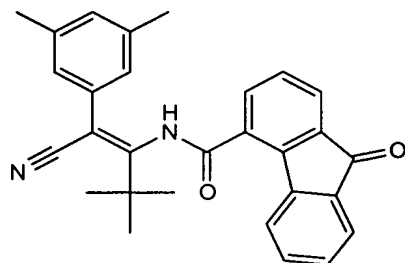
128-133

Compound 98



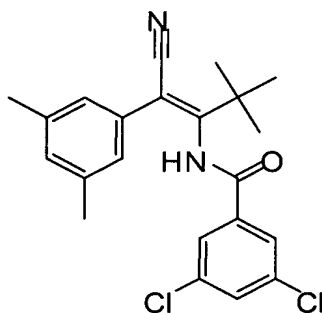
165

Compound 99



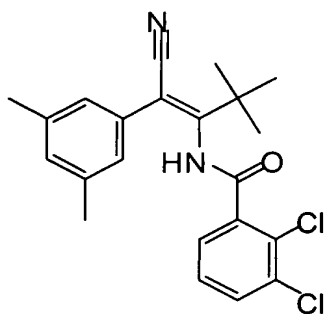
195

Compound 100



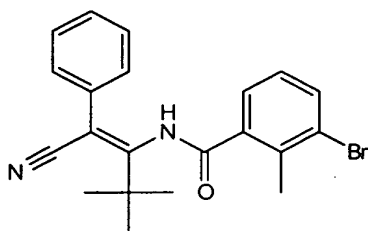
145

Compound 101



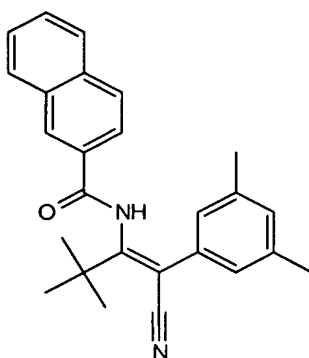
190

Compound 102



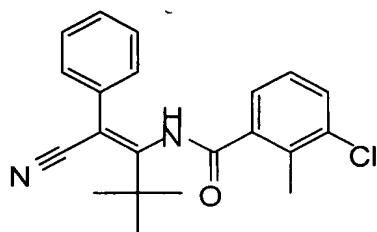
150

Compound 103



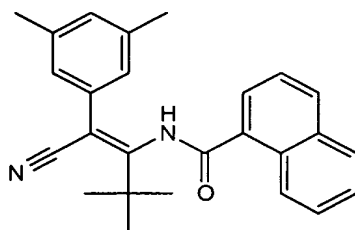
198

Compound 104



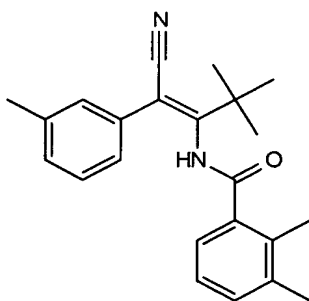
153

Compound 105



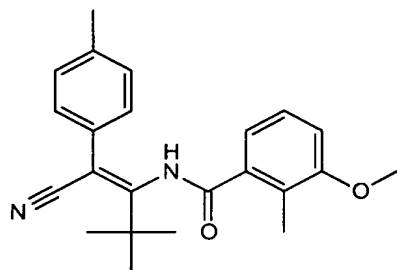
205

Compound 106



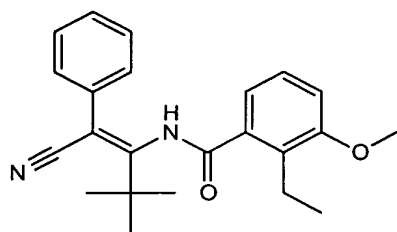
130

Compound 107



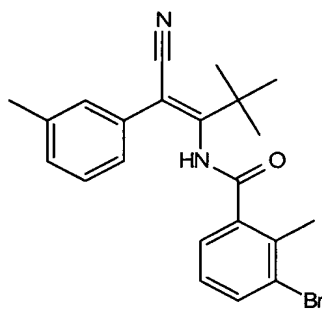
165

Compound 108



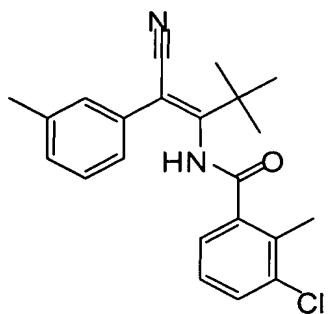
165

Compound 109



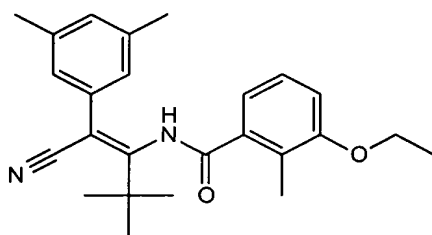
82.1

Compound 110



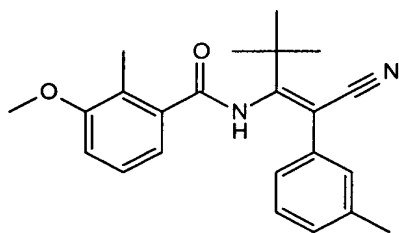
125

Compound 111



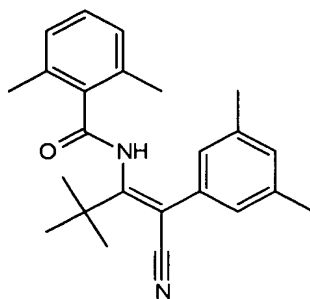
170

Compound 112



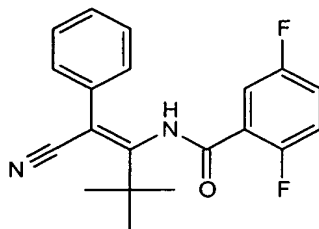
145

Compound 113



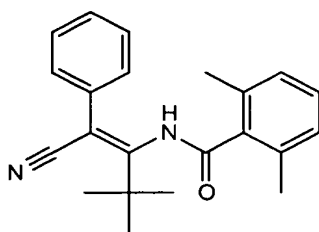
160

Compound 114



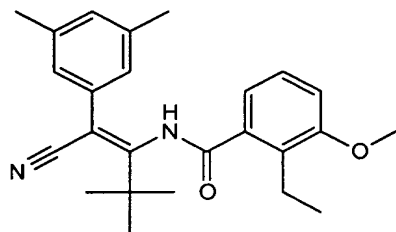
125

Compound 115



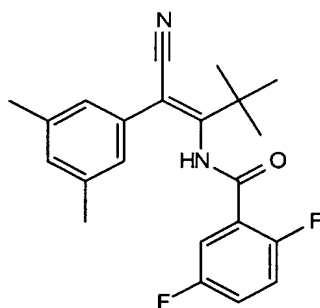
220

Compound 116



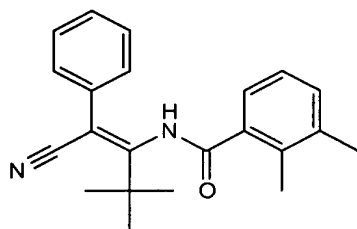
391

Compound 117



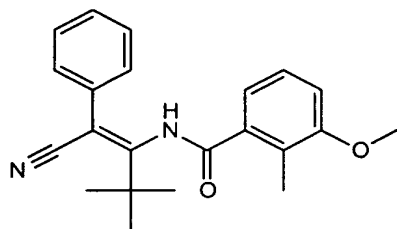
179-182

Compound 118



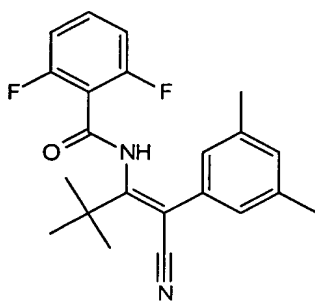
175

Compound 119



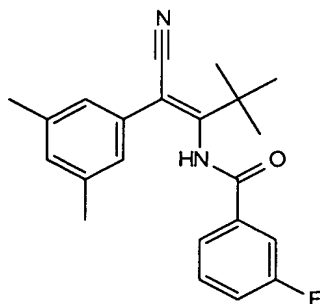
170

Compound 120



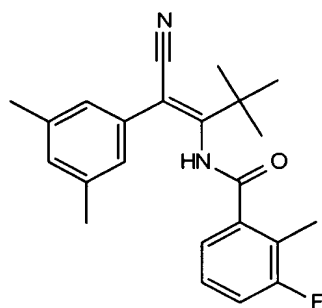
225

Compound 121



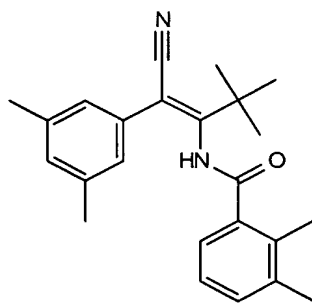
184.4

Compound 122



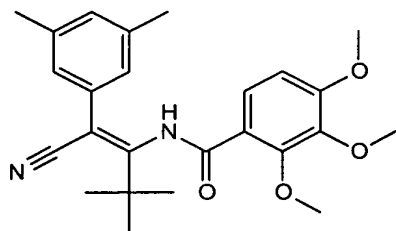
125.9

Compound 123



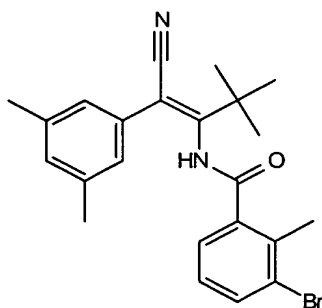
171-174

Compound 124



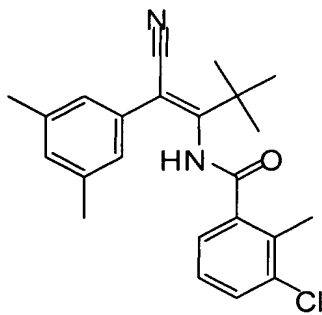
113.6

Compound 125



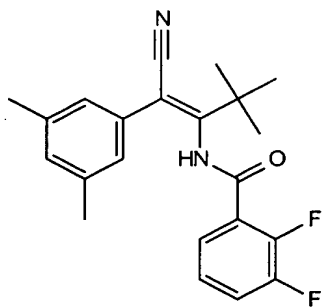
> 300

Compound 126



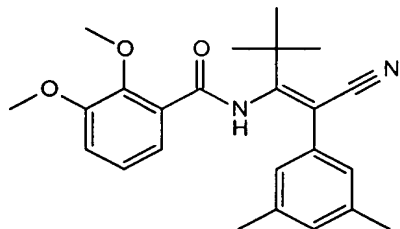
194.7

Compound 127



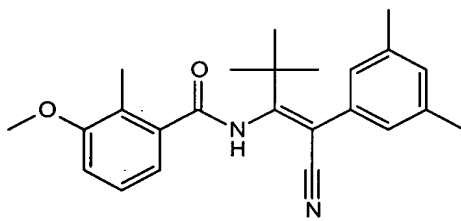
190

Compound 128



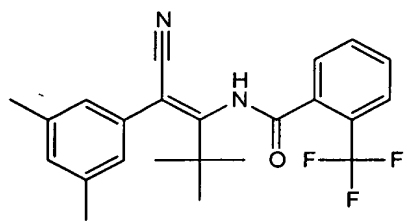
393

Compound 129



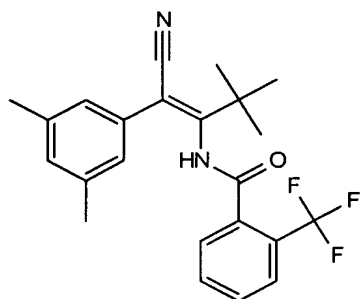
>300

Compound 130



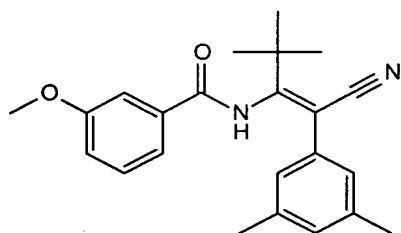
178-180

Compound 131



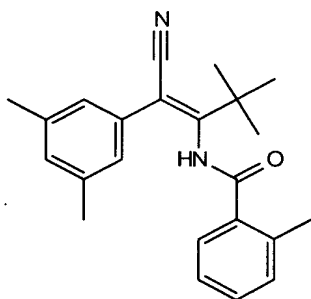
197-200

Compound 132



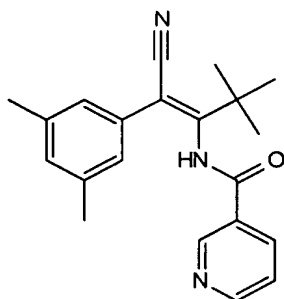
203-205

Compound 133



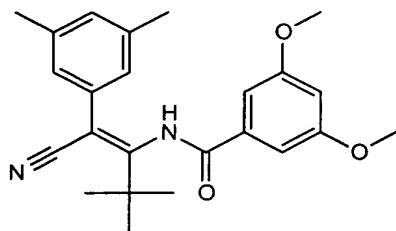
178-180

Compound 134



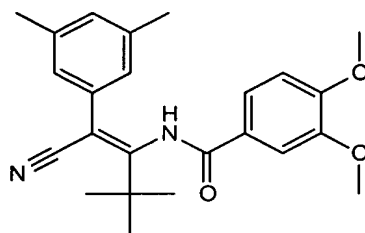
173-174

Compound 135



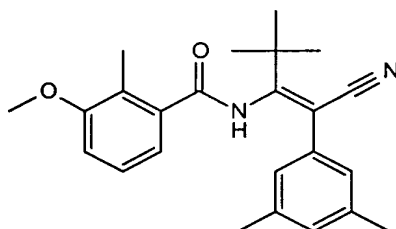
194-195

Compound 136



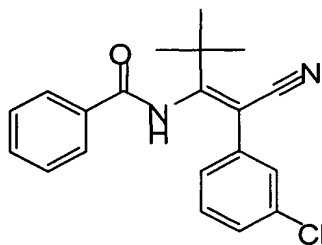
194-195

Compound 137



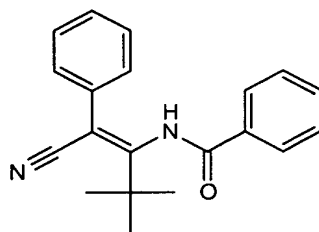
203-204

Compound 138



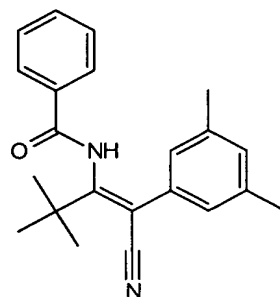
146-147

Compound 139



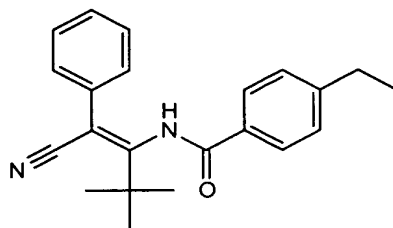
142-144

Compound 140



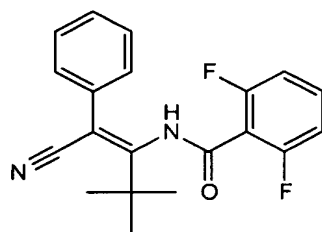
175-176

Compound 141



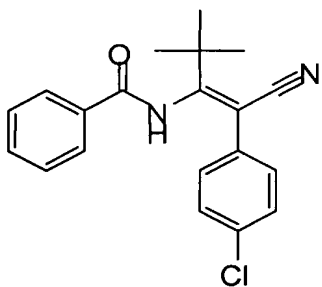
138-140

Compound 142



180-183

Compound 143



219-222

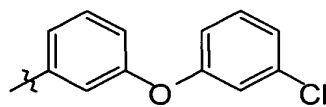
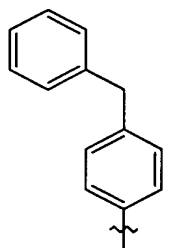
Compound 144

The various R1, R2, R3, and R4 moieties (from the compounds made as per Table A1 above) are summarized in Table A2 below.

Table A2

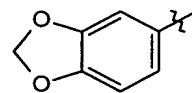
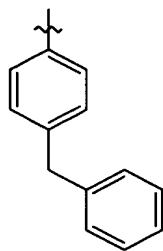
Compound #	R1	R2	R3	R4
1	t-Bu			H

6 t-Bu



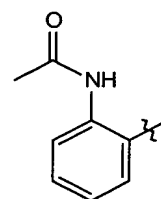
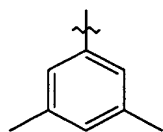
H

7 t-Bu



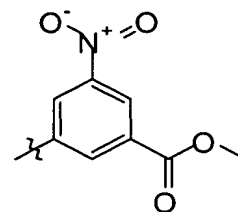
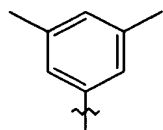
H

8 t-Bu



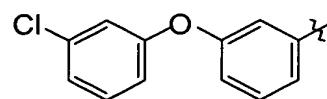
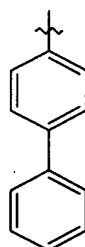
H

9 t-Bu



H

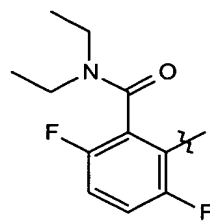
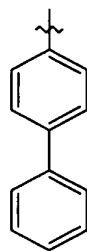
10 t-Bu



H

11

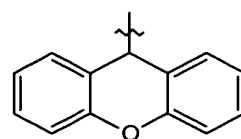
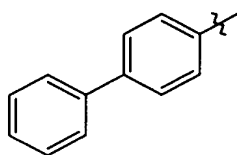
t-Bu



H

12

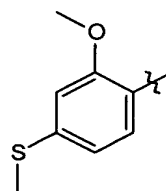
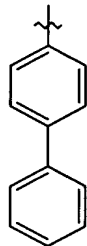
t-Bu



H

13

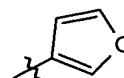
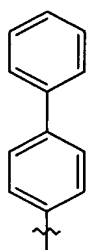
t-Bu



H

14

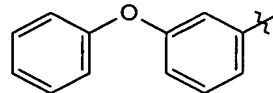
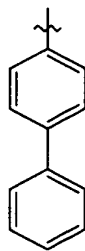
t-Bu



H

15

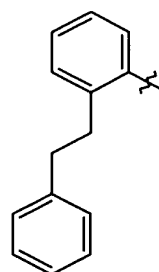
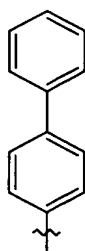
t-Bu



H

16

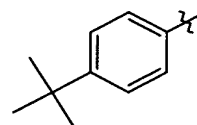
t-Bu



H

17

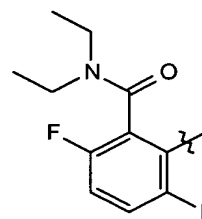
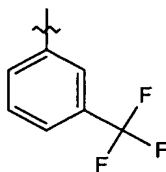
t-Bu



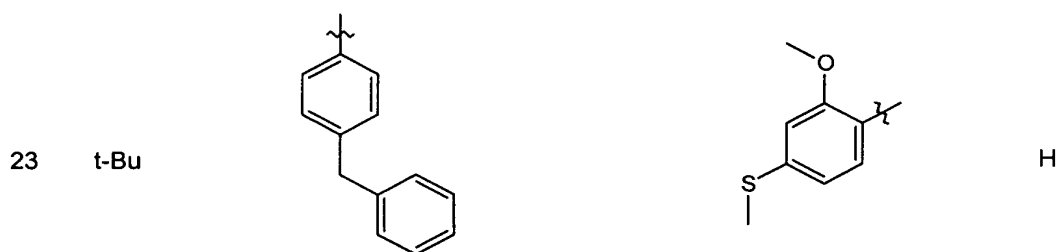
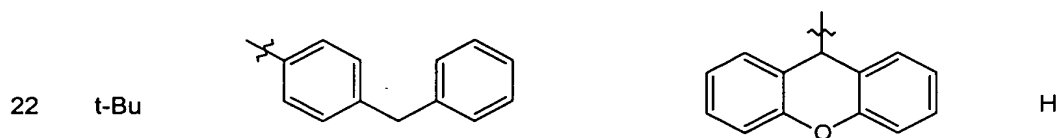
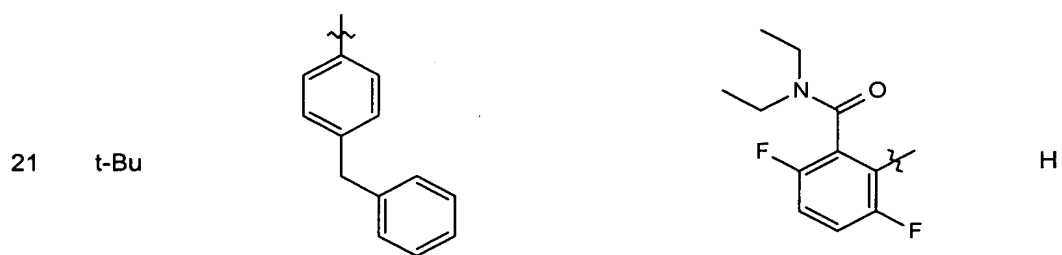
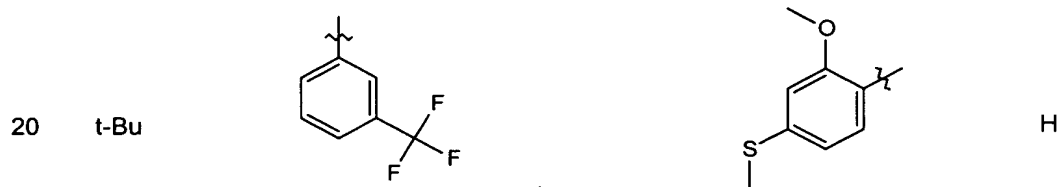
H

18

t-Bu

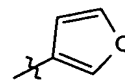
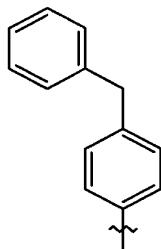


H



24

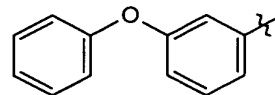
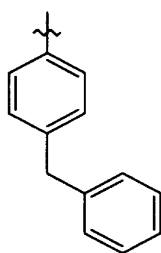
t-Bu



H

25

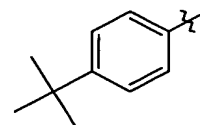
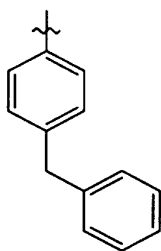
t-Bu



H

26

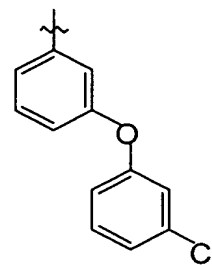
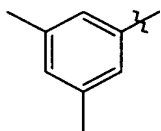
t-Bu



H

27

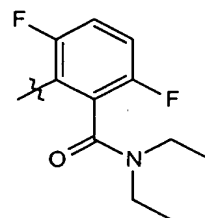
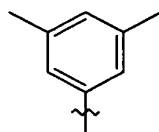
t-Bu



H

28

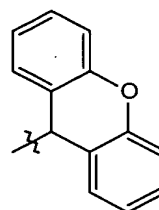
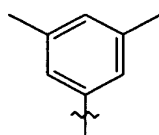
t-Bu



H

29

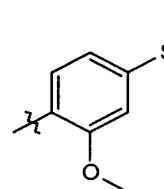
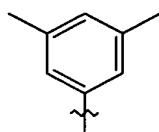
t-Bu



H

30

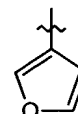
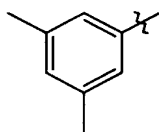
t-Bu



H

31

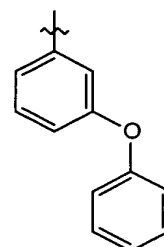
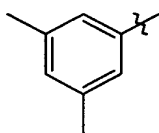
t-Bu



H

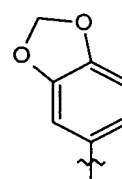
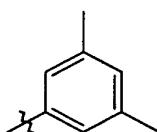
32

t-Bu



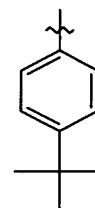
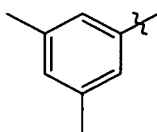
H

33 t-Bu



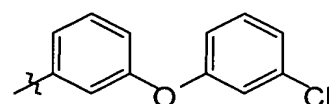
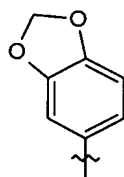
H

34 t-Bu



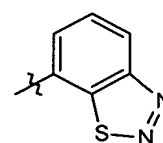
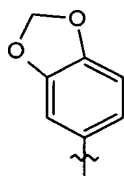
H

35 t-Bu



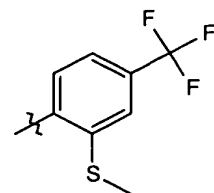
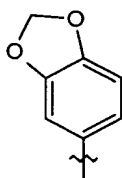
H

36 t-Bu



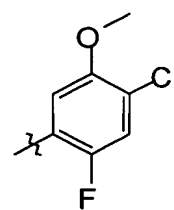
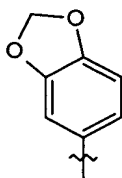
H

37 t-Bu



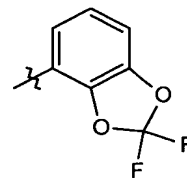
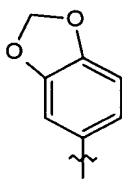
H

38 t-Bu



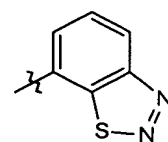
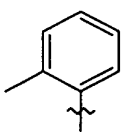
H

39 t-Bu



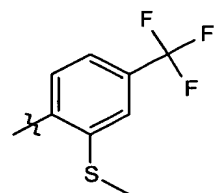
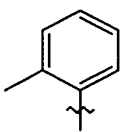
H

40 t-Bu



H

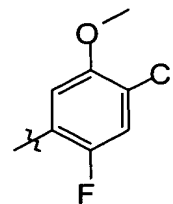
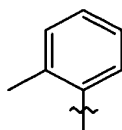
41 t-Bu



H

42

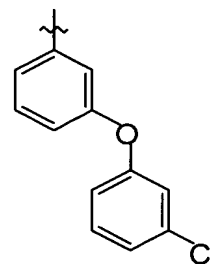
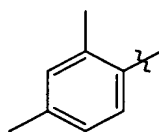
t-Bu



H

43

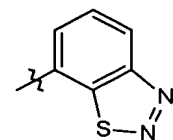
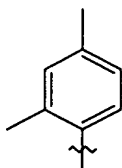
t-Bu



H

44

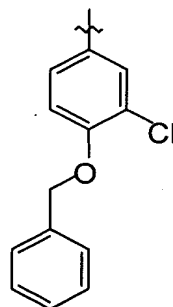
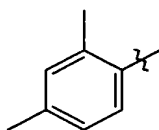
t-Bu



H

45

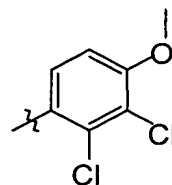
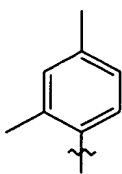
t-Bu



H

46

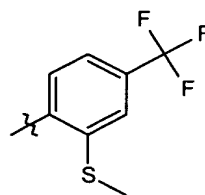
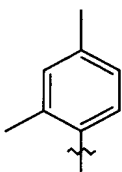
t-Bu



H

47

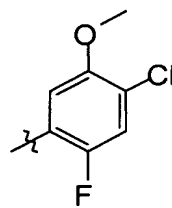
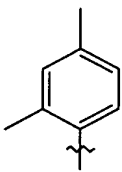
t-Bu



H

48

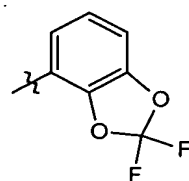
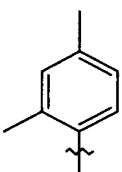
t-Bu



H

49

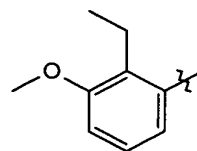
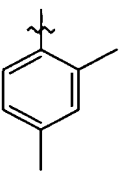
t-Bu



H

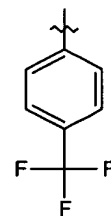
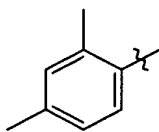
50

t-Bu



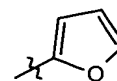
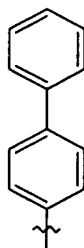
H

51 t-Bu



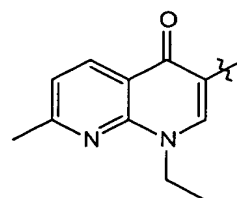
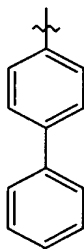
H

52 t-Bu



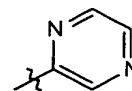
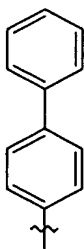
H

53 t-Bu



H

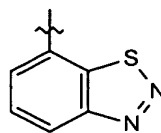
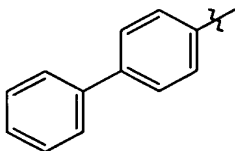
54 t-Bu



H

55

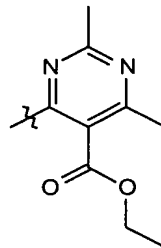
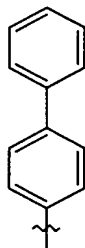
t-Bu



H

56

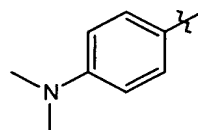
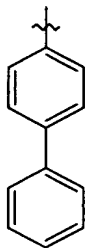
t-Bu



H

57

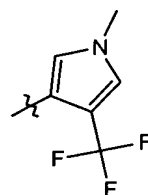
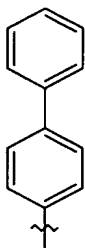
t-Bu



H

58

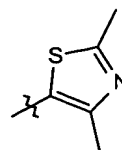
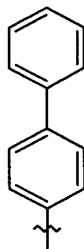
t-Bu



H

59

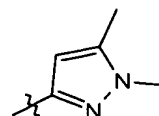
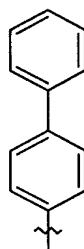
t-Bu



H

60

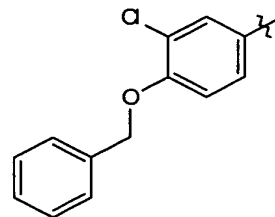
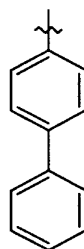
t-Bu



H

61

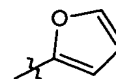
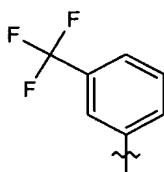
t-Bu



H

62

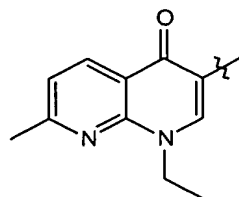
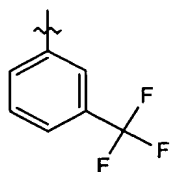
t-Bu



H

63

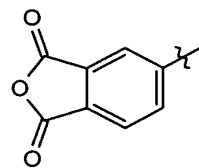
t-Bu



H

64

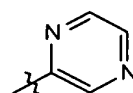
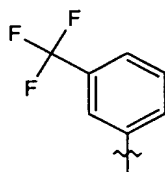
t-Bu



H

65

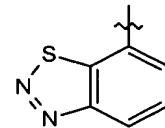
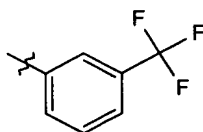
t-Bu



H

66

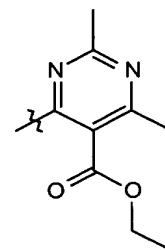
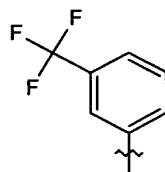
t-Bu



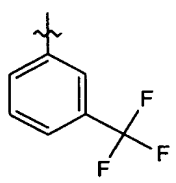
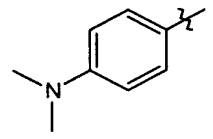
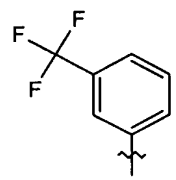
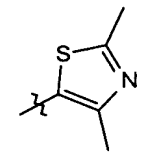
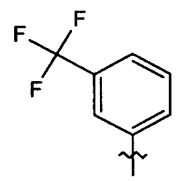
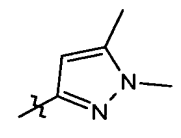
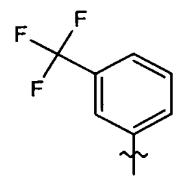
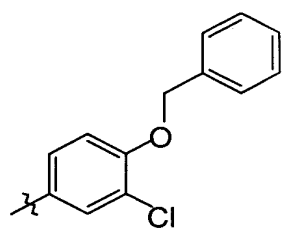
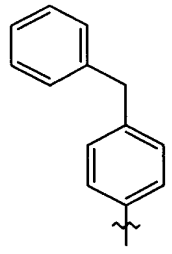
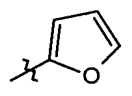
H

67

t-Bu

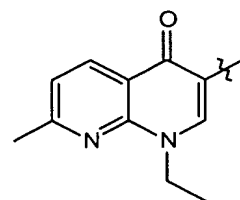
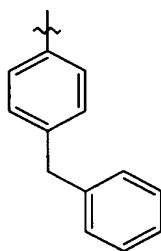


H

68	t-Bu			H
69	t-Bu			H
70	t-Bu			H
71	t-Bu			H
72	t-Bu			H

73

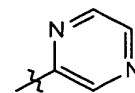
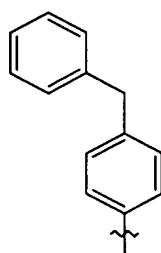
t-Bu



H

74

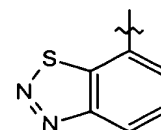
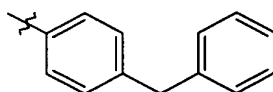
t-Bu



H

75

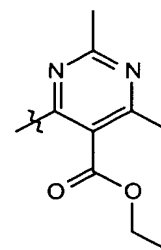
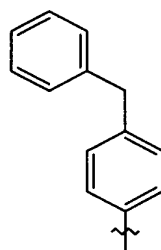
t-Bu



H

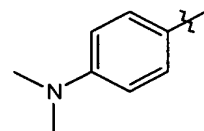
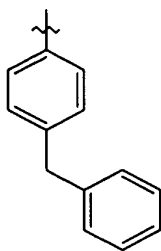
76

t-Bu



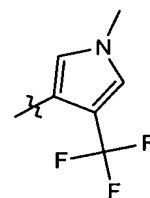
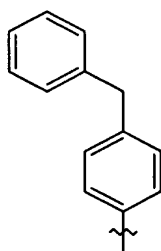
H

77 t-Bu



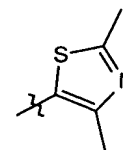
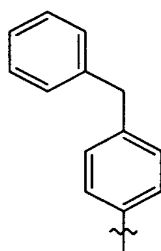
H

78 t-Bu



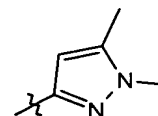
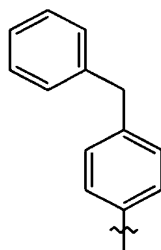
H

79 t-Bu



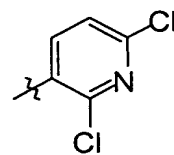
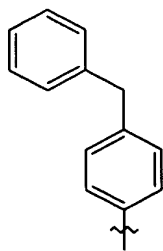
H

80 t-Bu



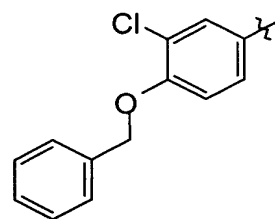
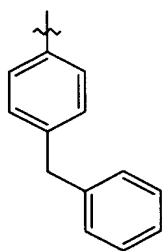
H

81 t-Bu



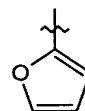
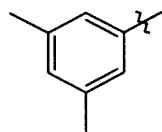
H

82 t-Bu



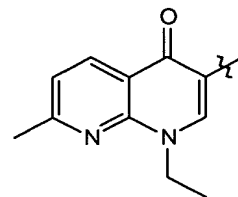
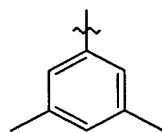
H

83 t-Bu



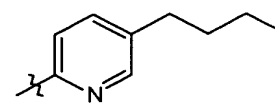
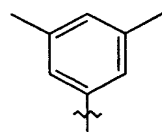
H

84 t-Bu



H

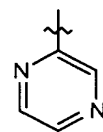
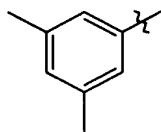
85 t-Bu



H

86

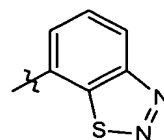
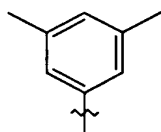
t-Bu



H

87

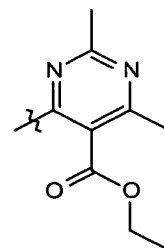
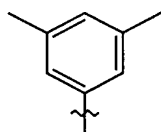
t-Bu



H

88

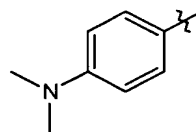
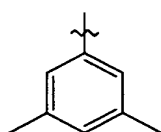
t-Bu



H

89

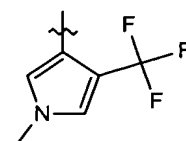
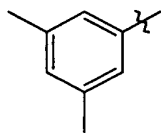
t-Bu



H

90

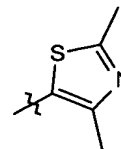
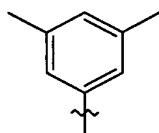
t-Bu



H

91

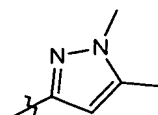
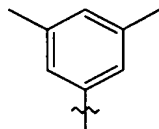
t-Bu



H

92

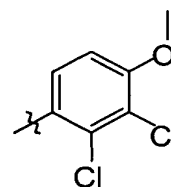
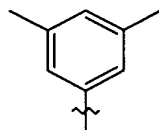
t-Bu



H

93

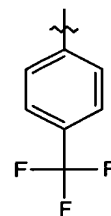
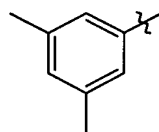
t-Bu



H

94

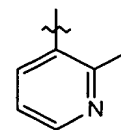
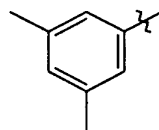
t-Bu



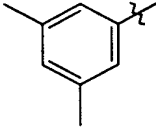
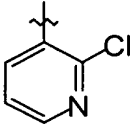
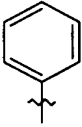
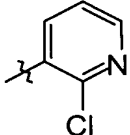
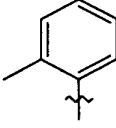
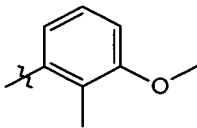
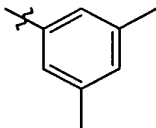
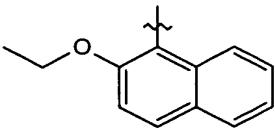
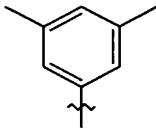
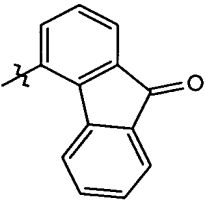
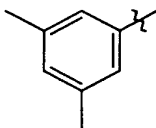
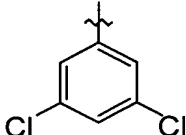
H

95

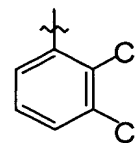
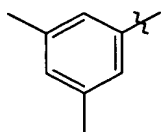
t-Bu



H

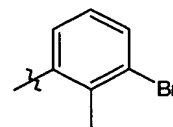
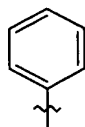
96	t-Bu			H
97	t-Bu			H
98	t-Bu			H
99	t-Bu			H
100	t-Bu			H
101	t-Bu			H

102 t-Bu



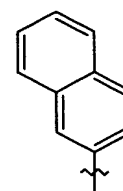
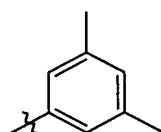
H

103 t-Bu



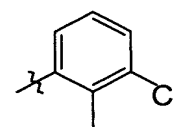
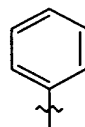
H

104 t-Bu



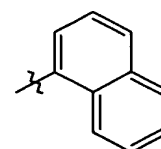
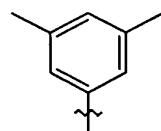
H

105 t-Bu



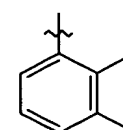
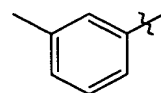
H

106 t-Bu

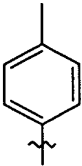
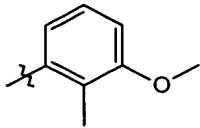
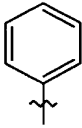
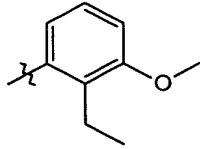
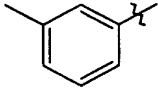
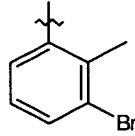
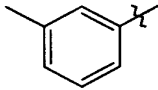
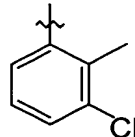
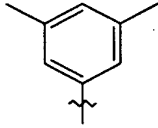
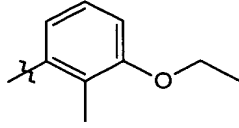
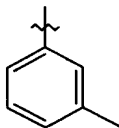
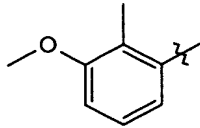


H

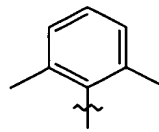
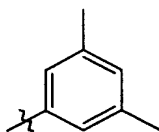
107 t-Bu



H

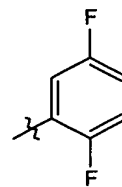
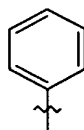
108	t-Bu			H
109	t-Bu			H
110	t-Bu			H
111	t-Bu			H
112	t-Bu			H
113	t-Bu			H

114 t-Bu



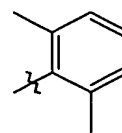
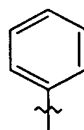
H

115 t-Bu



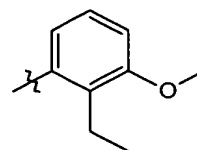
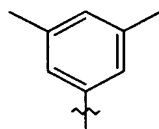
H

116 t-Bu



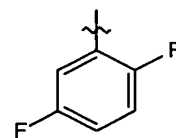
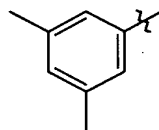
H

117 t-Bu



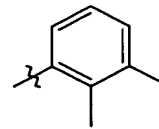
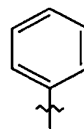
H

118 t-Bu



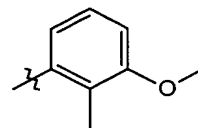
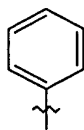
H

119 t-Bu



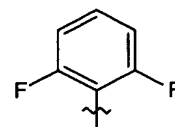
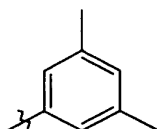
H

120 t-Bu



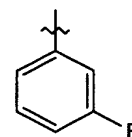
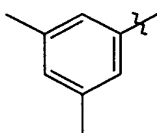
H

121 t-Bu



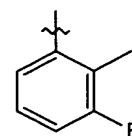
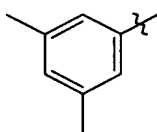
H

122 t-Bu



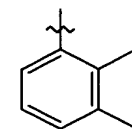
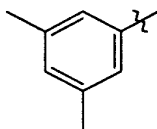
H

123 t-Bu



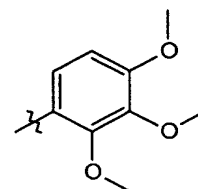
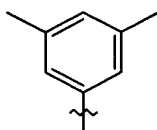
H

124 t-Bu



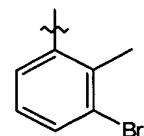
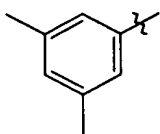
H

125 t-Bu



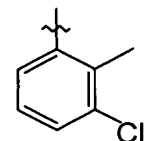
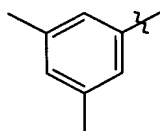
H

126 t-Bu



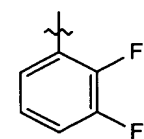
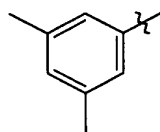
H

127 t-Bu



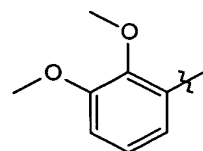
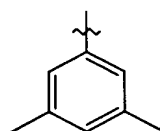
H

128 t-Bu



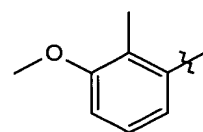
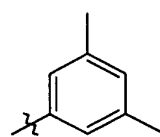
H

129 t-Bu



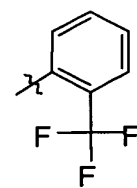
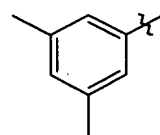
H

130 t-Bu



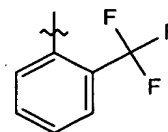
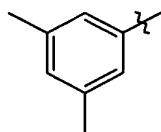
H

131 t-Bu



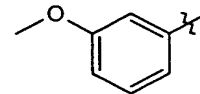
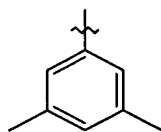
H

132 t-Bu



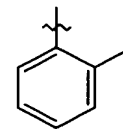
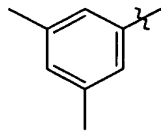
H

133 t-Bu



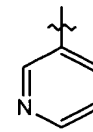
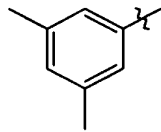
H

134 t-Bu



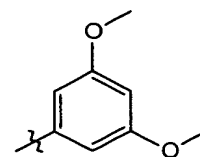
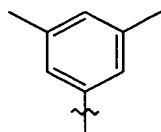
H

135 t-Bu



H

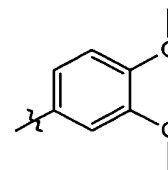
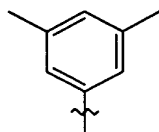
136 t-Bu



H

137

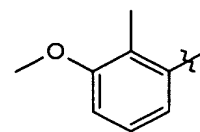
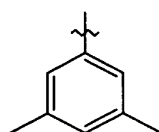
t-Bu



H

138

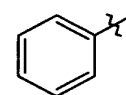
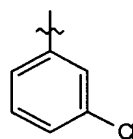
t-Bu



H

139

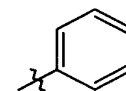
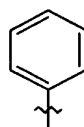
t-Bu



H

140

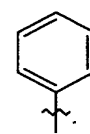
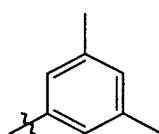
t-Bu



H

141

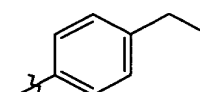
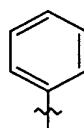
t-Bu



H

142

t-Bu

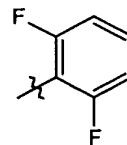
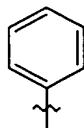


H

137 138 139 140 141 142

143

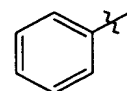
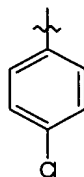
t-Bu



H

144

t-Bu



H

Example 3:

The following cyanoenamine compounds have been tested for pesticidal activity, according to the following procedures.

Spodoptera littoralis (abbreviated as SPODLI) (commonly known as Egyptian cotton leafworm): larvicide, feeding/contact activity. Cotton leaf discs were placed on agar in petri dishes and individually sprayed with each test solution of cyanoenamine in an application chamber. After drying, the leaf discs were infested with 20 to 25 L1 larvae. The samples were checked for mortality, repellent effect, feeding behavior, and growth regulation 2 and 6 days after treatment.

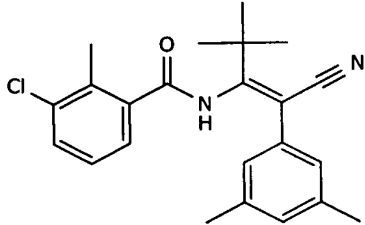
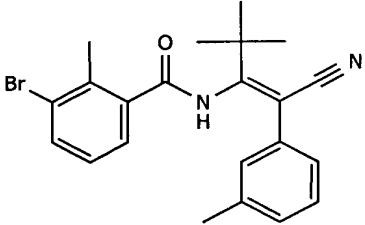
Heliothis virescens (abbreviated as Hv) (commonly known as tobacco budworm): ovo-larvicide, feeding/contact activity. 30 to 35 fresh eggs (0 to 24 hours old), deposited on filter paper, were placed in petri dishes on a layer of

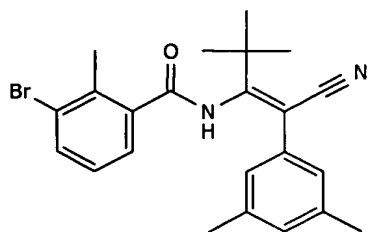
artificial diet and 0.8 mL of each test solution of cyanoenamine was individually pipetted onto them. After an incubation period of 6 days, samples were checked for egg mortality, larval mortality, and growth regulation.

Each of *Spodoptera littoralis* and *Heliothis virescens* is a larval form of
5 an insect in the order *Lepidoptera*.

The results are summarized in Table B below.

Table B

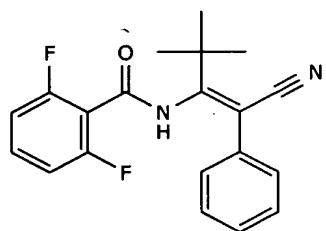
COMPOUND #	Insecticidal activity (EC80 ppm)	
	SPODLI	Hv
	50	50
Compound 127		
	200	200
Compound 110		



>50

>50

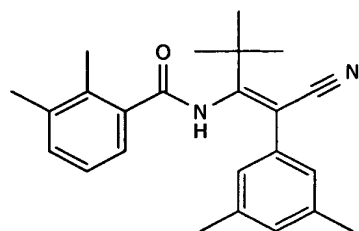
Compound 126



>>100

>100

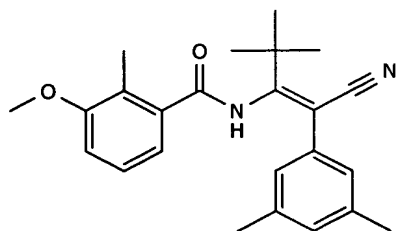
Compound 143



50

50

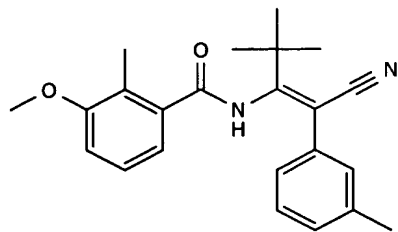
Compound 124



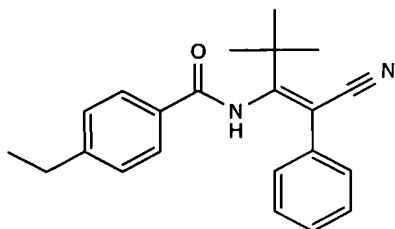
100

100

Compound 138



Compound 113



Compound 142



200

200

>>100

>>100



Example 4:

Construction of Reporter Plasmid

A minimal promoter vector was made by ligating a synthetic TATA box sequence oligonucleotide pair, 5'-agcttgagggtataatg-3' (SEQ ID NO:1) and 3'-actcccatattactcga-5' (SEQ ID NO:2), into the *Hind*III site of vector pGL3-basic (Promega) so that the *Hind*III site was recreated 5' to the inserted oligonucleotide and destroyed between the oligonucleotide and the downstream luciferase gene. This vector was designated TATA5.

The binding site from the hsp27 gene (Koelle *et al.*, *Cell* 67(1): 59-77 (1991)) was made with the oligonucleotide pair, 5'-gatccgagacaagggttcaatgcactgtccaatga-3' (SEQ ID NO:3) and 3'-gctctgttccaagttacgtgaacaggttactctag-5' (SEQ ID NO:4). This site was

multimerized and ligated into the *Bgl*III site of vector TATA-5. One isolate, pCGS154, contained the sequence below in the inserted region, having 2 pairs of sites in inverted orientations. One site had a deletion of a single base from the consensus sequence. The sequence of the inserted region in pCGS154 is

5 shown below:

```
1 gatccgagac aaggggtcaa tgcactgtc caatgagatc
41 cgagacaagg gttcaatgca cttgtccaat gagatctcat
81 tggacaagtg cattgaacct tgtctcggat ctattggac
121 aagtgcattg aaccctgtgc tcggatc (SEQ ID NO:5).
```

10

Cloning of EcR Receptor Plasmid

PCR primers were designed based on the published sequence for *Manduca Sexta* ecdysone receptor (EcR) (genbank accession number U19812 (SEQ ID Nos:6 and 7) to clone the gene in two halves. RNA was prepared from prepupae larva of *Manduca sexta* using the LiCl/phenol method (Current Protocols in Molecular Biology, Vol. 1, Unit 4.3, 1987, John Wiley and Sons, publishers) and 1 µg of total RNA was used to prepare cDNA using MMLV reverse transcriptase (Promega). The cDNA was used in a PCR reaction with the primers described above to generate two PCR products for the 5' and 3' halves of the gene. These were subcloned into the pGEM-TA vector (Promega) and sequenced. The two fragments were joined at a unique *Nde*I site within each fragment and ligated into pBS-KS (Stratagene) to create a full length *Manduca sexta* EcR clone named pBSFLMa. A *Hind*III site followed by

15

20

an inframe stop codon and *Bam*HI site was placed at the 3' end of the E domain (ligand binding domain) of the *Manduca* EcR receptor using the oligonucleotide: 5'-ggatcctaaagcttcgctcgtcgacacttcg-3' (SEQ ID NO:8).

5 A truncated *Manduca* EcR containing domains C, D and E of the receptor was constructed as follows. A *Bam*HI site and in-frame ATG was engineered just 5' to the C domain using the degenerate primers 5'-ggatccatgggycgagaagaatttcacccr-3' (SEQ ID NO:9) and 5'-ccacrtcccagatctcctcga-3' (SEQ ID NO:10). This fragment was then joined using the *Nde* site to the 3' end of *Manduca* EcR, which has an engineered *Hind*III
10 site at the 3' end as described above.

A fragment containing the herpes simplex VP16 transactivation domain was cloned from plasmid 35S/USP-VP16 (U.S. Patent No. 5,880,333) using the PCR primers 5'-aagcttgcccccccgaccg-3' (SEQ ID NO:11) (placing a *Hind*III site at the 5' end of the domain) and 5'-tctagaggatcctaccaccgtact-3' (SEQ ID
15 NO:12) (placing an inframe stop codon followed by *Bam*HI and *Xba*I sites at the 3' end of the domain). The VP16 domain was fused in frame to the 3' end of the E domain of the ecdysone receptor using the *Hind*III site 3' to EcR clone and the *Hind*III site engineered at the 5' end of VP16.

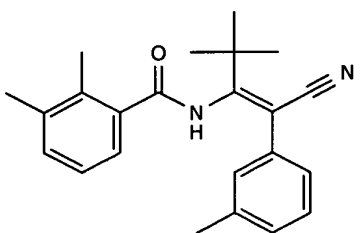
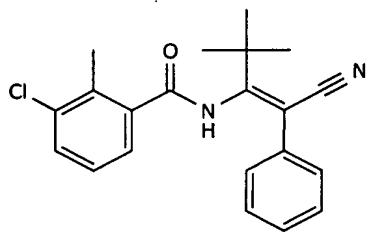
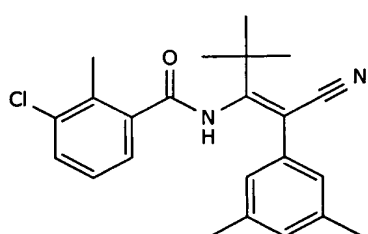
The plasmid pPacU (Courey AJ and Tjian R (1988) *Cell* 55, 887-898)
20 was used as the starting vector for expression constructs. The truncated *Manduca* EcR-VP16 was ligated into pPacU using the *Bam*HI sites flanking the coding region to create the construct referred to as MMV.

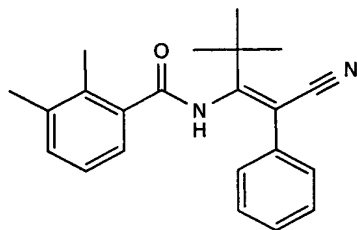
Cell-Based Assay

An *in vivo* cell based assay was used to measure transcriptional activation by the EcR receptor plasmid in the presence of the chemical ligands as described above. S2 *Drosophila* cells (ATCC CRL-1963) (commonly known as cells from the fruit fly) were transiently transfected with luciferase reporter (pCGS154) and receptor expression plasmid (MMV) using the calcium phosphate precipitation procedure (Di Nocera and David (1983) *PNAS* 80, 7095-7098). S2 cells were plated in 96 well format at a density of 2×10^5 in 166.6 μ l of Schneider's *Drosophila* media supplemented with antibiotics and 10% heat inactivated fetal bovine serum (GIBO-BRL). The following day, 33.4 μ l of a calcium phosphate precipitate containing 3-6 ng of pCGS154 reporter plasmid, and 3-6 ng of EcR receptor plasmid MMV along with salmon sperm DNA, to a total of 400 ng DNA per well were added. Chemical ligands (cyanoenamine test compounds) were added 16-24 hours after DNA addition to the cells at a final concentration of 2 μ M. Cells were then harvested and extracted 24 hours after chemistry addition following the procedures for the luciferase assay by centrifuging and resuspending the cell pellets in 100 μ l of cell culture lysis reagent (Promega). Luciferase activity was quantitatively determined using chemiluminescence (Promega) using an analytical luminescence model 2001 luminometer. Results were normalized as a ratio of induction relative to the reporter construct without chemical ligand addition.

The results are summarized in Table C below.

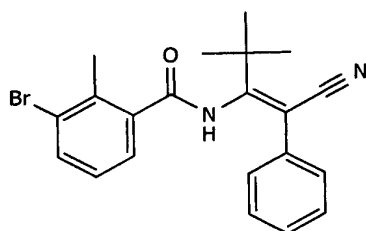
Table C

CHEMISTRY	Gene switch activity fold induction
 <p>Compound 107</p>	66
 <p>Compound 105</p>	191
 <p>Compound 127</p>	225



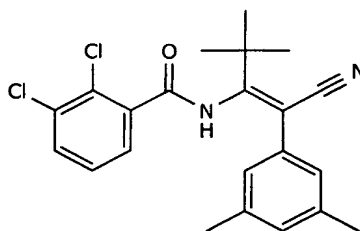
636

Compound 119



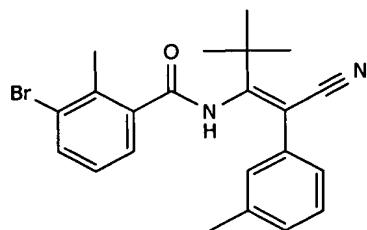
1112

Compound 103



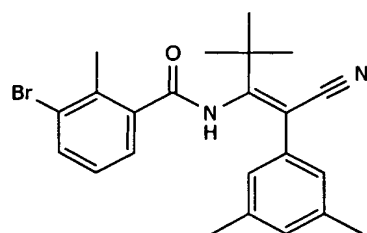
1145

Compound 102



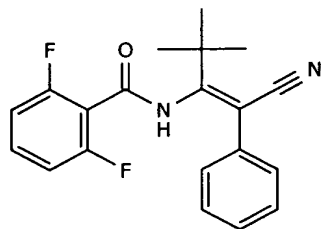
1012

Compound 110



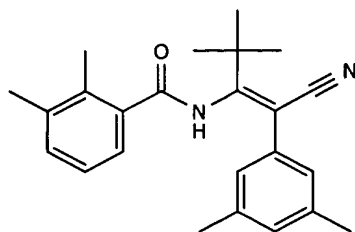
1121

Compound 126



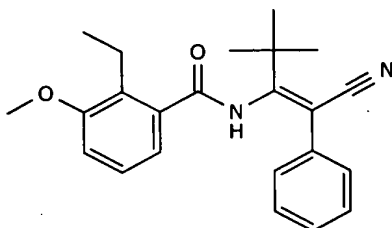
29

Compound 143



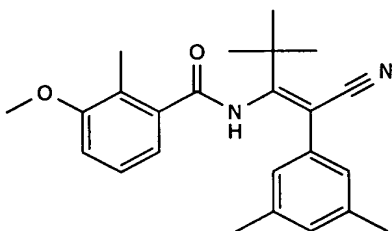
893

Compound 124



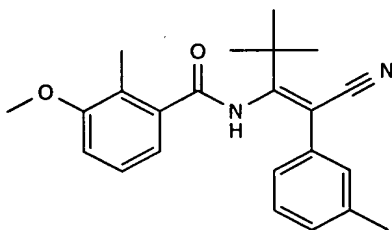
239

Compound 109



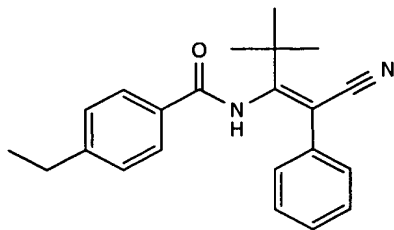
314

Compound 138



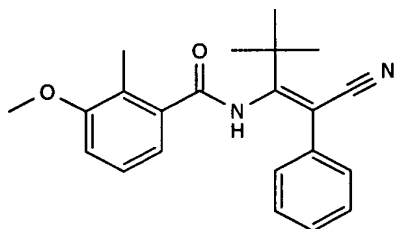
364

Compound 113



394

Compound 142



913

Compound 120

It will be understood that various details of the invention may be changed without departing from the scope of the invention. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation--the invention being defined by the claims.